Universitätsspital Zürich
Klinik und Poliklinik für Innere Medizin
Direktor: Prof. Dr. med. Edouard Battegay

Arbeit unter Leitung von:
Dr. rer. nat. Indranil Bhattacharya
(ab Februar 2009)
Prof. Dr. med. Matthias Barton
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Phosphorylation of Erf in Vascular Smooth Muscle Cells: Role of Erk-1/2 and Intracellular Calcium

INAUGURAL-DISSERTATION

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vorgelegt von
Stephanie Jane Verta-Nabholz
von Zürich

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Abstract

Ets-2 repressor factor (Erf) is a relatively little explored member of the Ets (E26 transformation specific)-domain transcription factor family. It is a phosphoprotein consisting of 548 amino acids, with a molecular weight of 75 kDa. Unphosphorylated Erf is a transcriptional repressor and induces cell cycle arrest in the G0/G1-phase, thus preventing cellular growth and proliferation. To present it is neither known whether endogenous Erf is expressed in vascular smooth muscle cells (VSMCs) nor how the phosphorylation of Erf is regulated in VSMCs. For this purpose A-10 embryonic rat thoracic aorta VSMC were treated with the mitogenic stimuli platelet-derived growth factor (PDGF) or low-density lipoprotein (LDL), and phosphorylation of Erf was analyzed by Western blot. Furthermore, to determine whether phosphorylation of Erf is dependent on extracellular signal-regulated kinase (Erk)-1/2, specific Mek-1/2 inhibitors U0126 and PD98059 were used. Additionally, phosphorylation of Erk-1/2 and Erf after treatment with the intracellular calcium chelator BAPTA/AM, either alone or in combination with PDGF, was investigated. The results show that Erf is expressed endogenously in VSMCs and phosphorylated by Erk-1/2 upon mitogenic stimulation. Furthermore, a decrease in intracellular calcium concentration, alone or in combination with a mitogenic stimulus, leads to increased phosphorylation of Erk-1/2 and Erf. These findings suggest that in VSMCs Erf phosphorylation is induced by pro-atherogenic agents (PDGF or LDL) via the activation of Erk-1/2 and regulation of Erf phosphorylation additionally involves levels of intracellular calcium. Thus, Erf could play an important role in VSMC proliferation, dedifferentiation and migration, and contribute towards the development of vascular remodelling diseases.

List of Abbreviations

AP-1	Activator protein 1
ATP	Adenosine-5'-triphosphate
BAPTA/AM	1,2-bis(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid Tetra(acetoxymethyl) ester
BSA	Bovine serum albumin
CAD	Coronary artery disease
CaD	Caldesmon
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
Ca^{2+}	Calcium ion with double positive charge
$[Ca^{2+}]_i$	Intracellular calcium concentration
CAP	Coronary artery plaques
CCB	Calcium channel blocker
Cdc2	Cell division cycle 2
CHD	Coronary heard disease
c-jun	Jun proto-oncogene (=AP-1)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemoluminiscence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Elk-1	Ets-like protein 1
eNOS	Endothelial nitric oxide synthase
Erf	Ets-2 Repressor Factor
Erk-1/-2 (p44/42)	Extracellular-signal-regulated kinases ½
ET-1	Endothelin 1
Ets-1/-2	E26 transformation specific 1/2
FCS	Fetal cow serum
HRP	Horseradish peroxidase
Jnk-1/-2/-3	c-Jun N-terminal kinases
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinases

Mek-1/-2	MAPK/ERK kinases
MHC	Myosin heavy chain
mi-RNA	Micro-RNA (ribonucleic acid)
MKK-1/-2	Mitogen-activated protein kinase kinases 1/2
MKKK	Mitogen-activated protein kinase kinase kinases 1/2
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_3VO_4	Sodium ortho-vanadate
NET-gelatine	Buffer with NaCl, EDTA and Tris
NO	Nitric oxide
NOS	Nitric oxide synthase
OV	Sodium Orthovanadate Na_3VO_4 (phosphatase inhibitor)
oxLDL	Oxidized LDL
PDGF	Platelet derived growth factor
PDGF-R	Platelet derived growth factor receptor
PBS	Phosphate buffer saline
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride (protease inhibitor)
$p21^{CIP}$	Cyclin-dependent kinase inhibitor
Ras	Rat sarcoma
A-/B-/C-Raf	Rapidly growing fibrosarcoma or rat fibrosarcoma A/B/C
RIPA	Radio-immuno precipitation assay
RTK	Receptor tyrosine kinases
SAPK	Stress-activated phospho-kinases
SDS-PAGE	Sodium-dodecyl sulphate – polyacrylamide gel electrophoresis
SMC	Smooth muscle cell
SM-1/-2/-22α	Smooth muscle-1/-2/-22α
TKI	Tyrosine kinase inhibitor
VRD	Vascular remodeling diseases
VSMC	Vascular smooth muscle cells

1. Introduction

1.1 Anatomy of an artery

Arteries can be divided into two different types, elastic and muscular arteries. Elastic arteries, including the aorta, have a more central location near the heart and fulfill the function of a blood reservoir, maintaining a constant blood flow. Muscular arteries, on the other hand, are located in the periphery and control the blood supply to the different organs by varying their diameter via nerval influence.[1, 2]

The arterial wall is made up of three layers – the tunica intima, tunica media and tunica adventitia (**Fig. 1**).[3, 4]

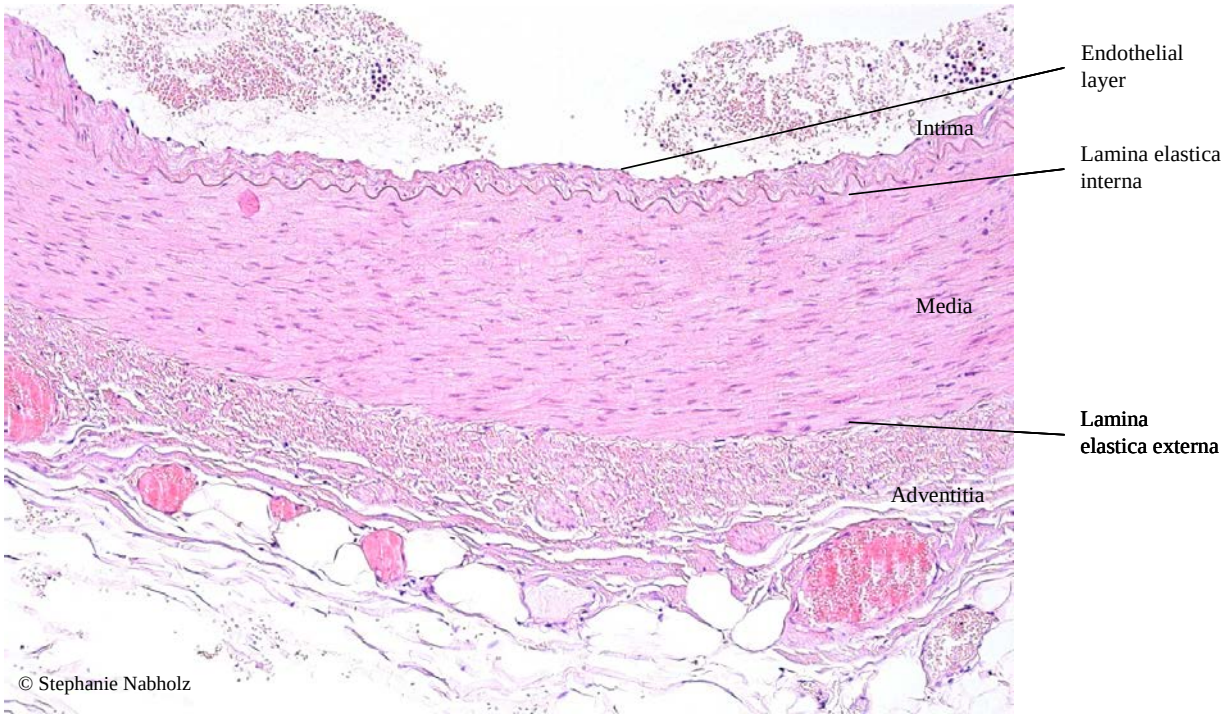

Fig. 1: Anatomy of a human muscular arterial wall. From the innermost to the outermost layer: tunica intima with the endothelial layer, lamina elastica interna, tunica media, lamina elastica externa, tunica adventitia. (Pictures: Institute of Pathology, Kantonsspital Baden, 2011).

As the innermost layer lining the lumen, the intima forms a barrier between the intravasal and the extravasal compartment of the body, preventing the loss of macromolecular substances and blood cells yet allowing the exchange of smaller molecules in specialized parts of the blood vessels.[3-5] The intima is made up of a single layer of endothelial cells as well as the

subendothelial space, a loosely-constructed reticular connective tissue containing fibroblasts and longitudinally-organized vascular smooth muscle cells (VSMCs).[1-4, 6] A band of elastic fibers, the lamina elastica interna, separates the intima from the media.[1-3, 6] It is less distinct in elastic arteries but very prominent in arteries of the muscular type. [4] The media is built up of multiple layers of VSMCs and elastic fibers arranged in concentric circles around the lumen of the vessel. Finally, the outermost part of the artery is the tunica adventitia, comprising elastic and collagen connective tissue, nerves and nerve endings of the vegetative nervous system and the vasa vasorum, small blood vessels for the supply of the vessel itself.[1-4, 6] The adventitia is separated from the media by the lamina elastica externa.[1-4]

1.2 Vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) are highly differentiated smooth muscle cells located within the walls of blood vessels, in particular in the media. VSMCs are spindle-shaped, often branched cells of approximately 20–300 µm in length with a centrally-located, longish nucleus (**Fig. 2**).[1, 3, 7, 8] In elastic arteries such as the aorta, the media contains several fenestrated layers of elastic fibers connected by VSMCs embedded in loose connective tissue.[2, 4] The VSMCs of subsequent layers are arranged in opposite directions to each other, thus permitting an adjustment in the thickness of the media depending on the momentary blood pressure.[4, 9] The media of muscular arteries on the other hand has more layers of VSMCs and only a few elastic or reticular fibers.[1-4, 10]

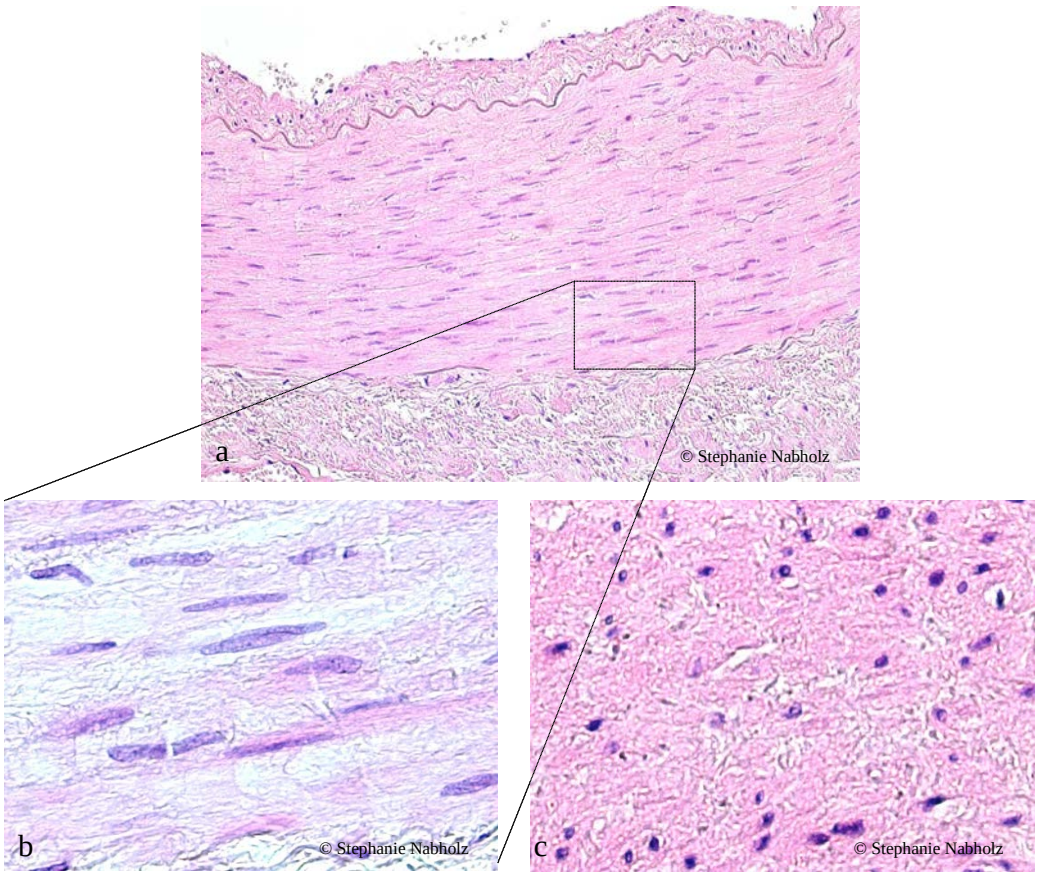

Fig. 2: Histological view of vascular smooth muscle cells in human muscular arteries. **a**: View of an arterial wall with the prominent media and the longitudinally-arranged VSMCs. **b**: Higher magnification of the VSMCs. **c**: Cross-section of the VSMCs of the tunica media; the nucleus has a round shape and is located in the center of the cell. (Pictures: Institute of Pathology, Kantonsspital Baden, 2011).

The VSMCs of an elastic arterial wall fulfill two main functions: 1) Regulating the distension of the arterial wall during the systolic phase.[4, 9] And 2) controlling the elastic recoiling of the arterial wall during the diastolic phase. These two mechanical processes help maintain constant blood flow in the blood vessels of the body (Windkessel effect).[6, 9-11] The VSMCs in muscular arteries and arterioles, however, regulate the diameter of the vessels via contraction (vasoconstriction) or relaxation (vasodilatation), thereby defining the blood flow per time unit in the vessel.[3, 10, 11] Looking at the circulatory system as a whole, a cumulative change in the total diameter of all the vessels not only affects blood flow but also blood pressure.[1, 3, 11]

1.2.1 VSMC markers

The VSMCs in a quiescent, differentiated state have very low rates of proliferation and express a specific set of protein markers as described below:

1. Smooth muscle α-actin

As a part of the contractile apparatus in differentiated VSMCs, smooth muscle α-actin is expressed at high levels. In differentiated VSMCs there are three other isoforms of actin (including non-muscle β-actin, non-muscle γ-actin and smooth muscle γ-actin). Smooth muscle α-actin accounts for over 40% of the total protein of a VSMC and for over 70% of the total actin. During vasculogenesis, the first marker expressed in developing and differentiating VSMCs is α-actin. As differentiation continues, the level of expression increases. Although smooth muscle α-actin is specific for smooth muscle, it can also be expressed in other cells such as myofibroblasts and endothelial cells upon stimulation by TGF-β.[12]

2. Smooth muscle myosin heavy chains

Smooth muscle myosin heavy chain (MHC) is another essential component of the contractile apparatus of a VSMC. In mature, differentiated VSMCs, there are at least three types of smooth muscle MHC, including SM-1 and SM-2, and two types of non-muscle MHCs. SM-1 and SM-2 are the two most specific markers for the identification of differentiated smooth muscle cells, especially SM-2, which is expressed in later stages of differentiation than SM-1.[12]

3. Myosin light chains

Aside from MHC, myosin contains a pair of non-phosphorylatable myosin light chains (MLCs) and a pair of regulatory, phosphorylatable MLCs. In VSMCs there are two non-phosphorylatable light chain isoforms, namely LC17a and LC17b, whereby higher levels of LC17b are associated with reduced ATPase activity in actin-myosin complexes. By

comparison, phosphorylatable MLCs exist in a number of isoforms in VSMCs, but also in numerous other cell types.[12]

4. Calponin and SM-22α

Calponin is said to have a regulatory function in the contraction of SMCs by interacting with F-actin and tropomyosin, as well as calmodulin. It has multiple isoforms, some of them also expressed in non-muscle cells. Together with SM-22α, the α- and β-isoforms of calponin are specific for adult SMCs. SM-22α is also one of the earliest markers in differentiated SMCs.[12]

5. Caldesmon

Caldesmon (CaD) can be found as two main isoforms in SMCs, h- and l-caldesmon. It binds to calmodulin and actin and is therefore thought to play a regulatory role in smooth muscle contraction. H-caldesmon is primarily expressed in SMCs and represents a marker for the intermediate stage of SMC differentiation, in comparison to l-caldesmon, which is also expressed in non-muscle cells.[12]

6. Tropomyosin

Tropomyosin (TM) is expressed in smooth muscle cells as well as in cardiac, skeletal and non-muscle cells and binds to the actin filaments of these cells. While it is essentially involved in the regulation of contraction in cardiac and skeletal muscle cells, it has a rather unclear function in SMCs. A stabilizing effect on actin has been postulated. The isoform α-TM is specific for SMCs in adult organisms.[12]

1.2.2 Contractile apparatus of VSMCs

The contractile apparatus of VSMCs consists of thick myosin filaments and thin actin filaments, so-called F-actin. Myosin filaments are polymers of the myosin-II molecule, a dimer consisting of two myosin heavy chains (MHC) forming the two heads and tail, and four myosin light chains (MLC) including two essential, non-phosphorylatable (ELC) and two regulatory, phosphorylatable MLC (RLC).[1, 13-15] An assembled myosin filament is

approximately 12-18 nm in diameter and contains up to 300 myosin heads.[13, 16, 17] F-actin on the other hand is a strand-like polymer of individual G-actin monomers with a diameter of approximately 5-8 nm.[1, 13, 16, 17] The thick filaments are arranged in parallel between the thin filaments, with the myosin heads pointing toward the actin filament on either side.[1, 8, 13] Myosin heads have an ATPase activity and the ability to cross link with actin.[1, 13, 14, 16, 17]

Associated with the actin filaments are, among other molecules, tropomyosin (TM), caldesmon (CaD) und calmodulin (CaM).[17] They represent the regulatory apparatus of smooth muscle cell contraction. In the unstimulated, relaxed state of the cell, caldesmon is closely associated with the actin-TM complex, thereby preventing cross-linking between actin and myosin and inhibiting ATPase activity of the myosin heads.[1, 13, 17]

The arrival of an external signal followed by the depolarisation of the cell membrane or the activation of G-protein coupled receptors (leading to the formation of inositoltriphosphate (IP3) via phospholipase C (PLC)) triggers an increase in intracellular calcium concentration, both due to influx through transmembrane L-type calcium channels as well as by release from the sarcoplasmatic reticulum (SR). The higher intracellular calcium concentration leads to the formation of calcium-calmodulin complexes which bind to caldesmon and also activate the myosin light chain kinase (MLCK).[8, 16, 17] Phosphorylation of the MLC by the MLCK, causing conformational change of the myosin heads, as well as exposure of the actin-binding site by dislocation of caldesmon enables actin-myosin cross-linking and finally smooth muscle cell contraction.[1, 8, 13, 16, 17] However, smooth muscle cell contraction is not only dependent on a rise in intracellular calcium concentration. It can also be induced by direct phosphorylation of calmodulin via the extracellular signal-regulated kinases (Erks), thus breaking the bonds between calmodulin and actin.[17]

1.2.3 Diseases involving VSMCs

Vascular diseases can affect every layer of the vessel wall and cause functional and structural changes particularly in the endothelium and the intima, but also in the VSMCs of the media.[3, 5] By taking a closer look at the development of the most common angiopathy, atherosclerosis, the impact of the changes in VSMCs on the architecture of the vessel wall becomes apparent.

In industrial countries, atherosclerosis and associated complications such as coronary heart disease (CHD)/coronary artery disease (CAD) have become the number one cause of death. Risk factors involve hypertension, hypercholesterolemia, diabetes mellitus, obesity, male gender, metabolic syndrome as well as systemic inflammatory diseases. Moreover, environmental factors including nicotine abuse, high-fat diet and lack of adequate exercise also contribute to the development of this vascular disease.[3, 18] The genesis of an atherosclerotic plaque, however, is not dependent on one of these factors only, but is almost always multifactorial.[3, 18, 19]

The response-to-injury hypothesis [3, 20, 21] in combination with the lipid hypothesis [22] poses a possible theory on how atherosclerotic plaques can develop in elastic and muscular arteries.[3, 19] Mechanical stress such as hypertension, as well as inflammation, damage the vessel wall and in particular the innermost layer, leading to endothelial dysfunction.[3, 20] In the initial stage this causes increased endothelial permeability with influx of plasma-derived cholesterol in form of lipoproteins such as low-density lipoproteins (LDL) into the intima.[3, 18, 20] Reactive oxygen species (ROS) and other free radicals such as nitric oxide (NO), produced by the surrounding cells of the vessel wall, lead to the oxidation of LDL.[3, 18, 23] Oxidized LDL (oxLDL) stimulates the expression of cytokines (e.g. tumor necrosis factor, TNFα), vasoactive substances (e.g. angiotensin II) and growth factors (e.g. platelet derived growth factor, PDGF) in endothelial cells and promotes their release into the intra- and extravasal compartment, thereby activating VSMCs in the media.[3, 18, 24] LDL and oxLDL, however, can also stimulate the VSMCs directly.[25]

By release of chemoattractant molecules and cytokines the endothelial cells are able to attract monocytes in the blood and permit them to transmigrate into the vessel wall.[3, 18] Stimulated by the inflammatory milieu, the monocytes undergo transformation to macrophages that take up the oxidized LDL.[3, 18, 20, 21, 23] The local inflammatory process eventually turns into a chronic systemic inflammatory reaction.[3, 18, 21] Once the macrophages are loaded with oxidized LDL, they turn into foam cells, initially leading to the formation of subendothelial fatty streaks.[3, 18-20, 23] With further progression of atherosclerosis, the foam cells die or undergo programmed cell death, freeing the phagocytosed lipids and generating a necrotic fatty core of the growing atherosclerotic lipid plaque.[3, 18, 20] Interestingly, not only macrophages undergo this process; VSMCs can also take up oxidized LDL and turn into foam cells.[18, 19, 23]

Vascular injury in general and atherosclerosis in particular cause VSMCs to migrate to the intima where they lose their differentiation markers such as α-actin, smooth muscle MHC, caldesmon and desmin.[5, 12, 18, 23 26-29] These changes are associated with an increased proliferation of VSMCs [3, 5, 18, 26, 29, 30] as well as production of extracellular matrix components including collagen, elastin and glycosaminoglycans (GAG),[1, 3, 18, 26, 31] ultimately causing intimal swelling and vascular remodelling.[3, 5, 18, 20, 21, 23, 24, 26, 31]

1.3 Cell signaling in VSMC

Vascular smooth muscle cells express both intracellular/nuclear and cell surface receptors. There are three primary forms of cell surface receptors: ion-channel-linked receptors, G-protein coupled receptors and enzyme-linked receptors including the receptor tyrosine kinases (RTKs).[13, 32-35] The mechanisms behind transduction of a signal from the extracellular to the intracellular space include a conformational change of the receptor, activation of the coupled G-protein and dimerization of the receptor followed by autophosphorylation.[13, 34-36] For example, the simultaneous binding of the dimeric platelet-derived growth factor (PDGF) to

two PDGF receptor tyrosine kinases leads to their dimerization and autophosphorylation at tyrosine residues.[36] One of the most common proteins activated by receptor tyrosine kinases is the monomeric GTP-binding protein Ras.[13, 37] The extracellular signals transmitted by the receptors activate downstream kinases which frequently converge at mitogen-activated protein kinases (MAPKs).[36, 37] Activation/deactivation of signaling elements takes place primarily by the process of phosphorylation or dephosphorylation of amino acids of the target molecule at serine, tyrosine and threonine residues.[13, 37-39]

1.3.1 Role of mitogen-activated protein kinases

The mitogen-activated protein kinase (MAPK) pathway is one of the most essential signaling pathways of many cells, including VSMCs.[38-40] Stimulated by mitogens including growth factors, hormones and cytokines, it regulates important cell functions such as proliferation, gene expression, cell metabolism, differentiation and apoptosis.[36-39, 41] The pathway constitutes a phosphorelay system of three sequentially phosphorylated and activated protein kinases.[37-39] The starting point of the entire phosphorylation cascade are the MAPK kinase kinases (MKKKs). There are several forms of MKKKs so that the various signals a cell receives can be transmitted to the corresponding pathways, thus leading to an adequate reaction of the cell to the requirements of its changing environment.[38, 39, 41] The MKKKs pass on the signal by phosphorylation of the MAPK kinases (MKKs). In the end the phosphorylation cascade leads to the activation of the MAPKs, which are serine/threonine-specific protein kinases.[36-39, 42] This relay system allows the cell to amplify the transmitted signal and also enables regulatory interventions by other signaling pathways.[36, 38]

The best-researched and best-documented MAPKs are the extracellular signal-regulated kinases (Erks) and the family of stress-activated protein kinases (SAPKs), including the c-Jun N-terminal kinases (Jnks) and the p38s.[36, 37, 39]

The Erk-MAPK pathway is put into action by extracellular stimuli, including growth factors and cytokines, through activation of the proto-oncogene Ras (**Fig. 3**).[36, 37, 39, 41] Ras is a G-protein with the ability to pass on a signal to the proto-oncogenes raf A/B/C (c-Raf1, A-Raf and B-Raf), the MKKKs of the signaling cascade.[36-38, 41] While A-Raf and B-Raf are restricted to certain tissues and fulfill more specific functions, Raf-1 is expressed ubiquitously.[38] Apart from the activation by Ras, Raf is additionally regulated via negative feedback inhibition.[38] Following arrival of a signal, Raf activates Mek-1 and Mek-2 (the MKKs) by phosphorylation at two serine sites.[36-38, 41] Finally, Mek-1 and Mek-2 phosphorylate Erk-1 and Erk-2 (alias p44 and p42 MAPKs, corresponding to their molecular weights) at tyrosine and threonine residues.[36-38, 41-43] Erk-1/2 represent serine/threonine protein kinases with more than 50 substrates [38, 39, 43] involved in increased proliferation and/or changes in post-mitotic activity.[36-39, 43]

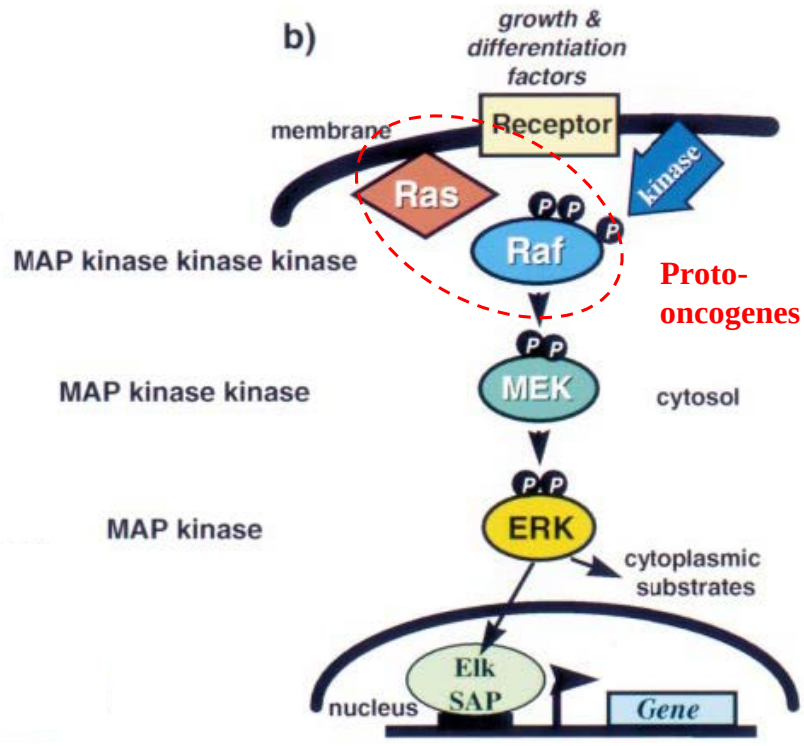

Fig. 3: Schematic picture of the Ras-Raf-Mek-Erk signaling pathway. The extracellular signal molecule binds to the receptor protein. In the case of the Erk-pathway the G-protein is Ras. The G-protein passes the signal on to the downstream signaling proteins, which in turn modulate the target proteins (e.g. transcription factors). Framed by the red dashed circle are the two proto-oncogenes Ras and Raf. (Adapted from: Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem. J*. 2000; **351**: 289-305).

Erk-2 (p42) has been shown to be phosphorylated during the G1 phase of the cell cycle of Chinese hamster ovary cells following a mitogenic stimulus.[44] During mid to late G1 phase it then shuttles to the nucleus, where it is necessary for cell cycle progression and consequently proliferation.[44] For this reason, members of the Ras-Raf-Mek-Erk pathway are being discussed as potential targets in the treatment of certain types of cancer.[37, 45]

In contrast to Erk-1/2, SAPKs (such as Jnks) are activated by inhibition of protein synthesis and other stress-triggering events via several different MKKKs.[37, 39, 46] The activated Jnks phosphorylate and bind the c-Jun protein, an element of the activator protein 1 (AP-1) transcription complex that regulates gene transcription (**Fig. 4**).[39, 46]

Further members of the SAPK family are the p38 kinases. The four p38 kinases α, β, γ and δ are part of the signaling pathway activated by inflammatory cytokines including interleukins and hormones, and stress factors such as osmotic shock and heat shock (**Fig. 4**).[37, 39] Therefore, p38 kinases play an important role in immune response and the development of autoimmune diseases, for example asthma, by regulating the expression of inflammatory cytokines.[37, 39, 46]

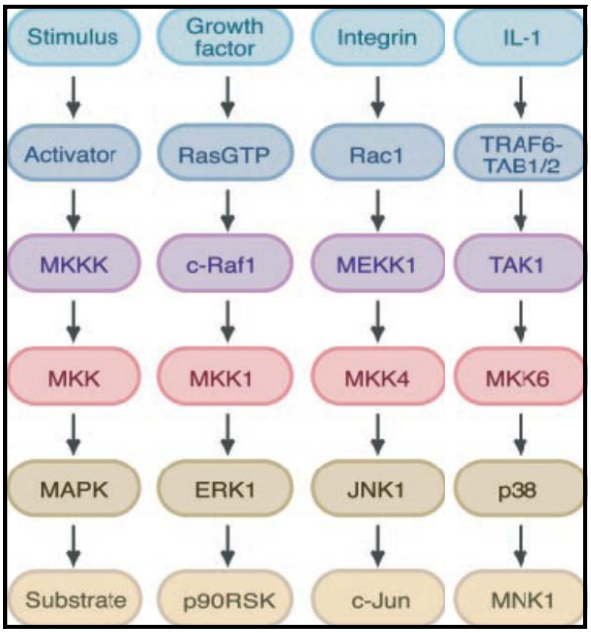

Fig. 4: MAPK pathways. The first lane shows the pattern of the pathway. The second lane shows the Erk-1/2 activation pathway. The third lane shows the Jnk activation pathway. The last lane shows the pathway involving activation of p38. (Adapted from: Johnson GL, Lapadat R. Mitogen-Activated Protein Kinase Pathways Mediated by Erk, Jnk, and p38 Protein Kinases, *Science.* 2002; **298**: 1911).

In VSMCs the activation of the MAPK pathways through mitogens such as platelet derived growth factor (PDGF) or low-density lipoproteins (LDL) has been specifically shown to induce migration and/or cell proliferation as well as regulation of contractile phenotype.[24, 47-57] Activation of the MAPK pathway and specifically Erk-1/2 can also induce upregulation of smooth muscle MHC expression in neonatal rat VSMCs, thus allowing dedifferentiated VSMCs to regain contractile function.[29]

1.3.2 Role of calcium

Calcium is an important intracellular ion involved in multiple VSMC processes.[58, 59] Under unstimulated, physiological conditions in muscle cells, intracellular calcium concentration $[Ca^{2+}]_i$ is tightly regulated and kept at 100 nM.[11] It is stored in the so-called sarcoplasmatic reticulum as well as in the caveolae along the cell membrane of VSMCs.[1, 58, 59] Following nerval stimulation by the vegetative nervous system (e.g. via α1-adrenoceptors) as well as stimulation by extracellular signals (e.g. LDL, PDGF, angiotensin II and vasopressin), Ca^{2+} is released from the sarcoplasmatic reticulum into the cytoplasm as a result of inositol triphosphate (IP_3) formation.[1, 58-66] In addition there is Ca^{2+}-influx from the extracellular space via L-type calcium channels due to depolarization of the cell membrane.[1, 13, 58, 59, 65, 67] This rise in $[Ca^{2+}]_i$ ultimately triggers smooth muscle cell contraction.[1, 13, 58, 59, 65]

However, Ca^{2+} not only plays a role in smooth muscle cell contraction, it is also an important mediator and regulator of intracellular signal transduction. Kip et al., showed that reduction of $[Ca^{2+}]_i$ in VSMCs by the calcium ion antagonist Verapamil or the Ca^{2+}-chelator BAPTA/AM induces production of the intracellular second messenger cyclic adenosine monophosphate (cAMP) as well as increased Erk-2 signaling and proliferation of VSMCs in adult mice thoracic aortas.[68] Furthermore, it has been reported that in human aortic VSMCs, reduction of $[Ca^{2+}]_i$ induces proliferation due to activation of the MAPK pathway.[69]

1.4 Ets-domain transcription factor family

Transcription factors are proteins with DNA-binding domains. By binding to specific DNA regions they regulate the transcription of target genes, thereby affecting cell differentiation, proliferation and metabolism.[70, 71]

The Ets-domain (E26 transformation-specific sequence of the avian erythroblastosis virus) transcription factor family represents a large group of transcription factors expressed partly ubiquitously, partly tissue-specifically.[26, 71] Members of the family include Ets-1, Ets-2, Erf, Fli-1 and the ternary complex factor (TCF)-sub family with Elk-1.[71-73] They are involved in a multitude of cell functions such as cellular growth, proliferation and differentiation, angiogenesis, hematopoiesis, but also in vascular inflammation, remodelling and apoptosis.[26, 70-76] As proto-oncogenes they also play an important role in the development of various solid and haematological cancer types.[26, 71, 74, 76]

With the exception of Elk-1, SAP-1 and Elf-1, the members of the Ets family share a conserved sequence of 85 amino acids.[72, 73, 75, 76] The DNA-binding domain of the Ets-domain transcription factors constitutes a variation of the winged helix-turn-helix motif and binds to gene sequences with a central GGAA/T-motif.[26, 70-73, 75, 76] Interactions with specific co-regulatory proteins can alter DNA-binding properties and increase the specificity of promoter recognition. This enables a more precise control of gene expression.[70-73, 76]

Along with a multitude of signaling pathways, the Ets-domain transcription factors are regulated by the MAPK pathway in particular,[71-73, 75-79] including the Erk-1/2 and the SAPK pathways (Jnk1 and p38) (**Fig. 5**).[71-73, 76, 77] Phosphorylation of Ets-domain transcription factors causes conformational change or a alterations in the subcellular localization, leading to enhanced or decreased protein-co-regulator and protein-DNA–binding and, thus, affecting transcriptional activity.[26, 71, 72, 76] In the case of Ets-2-repressor factor (Erf), phosphorylation leads to the export of the protein from the nucleus to the intracellular space and thus to limitation of its repressor activity.[71, 76, 77] Ets-1, Ets-2 and Erf, as well as Elk-1, are explicit

direct targets of the extracellular signal-regulated MAPK pathway (Erk-MAPK pathway).[72, 77-79]

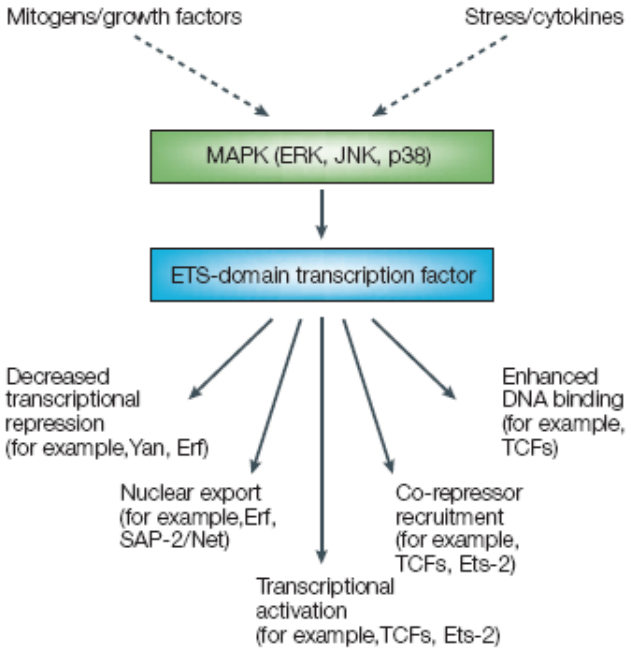

Fig. 5: Schematic representation of Ets-domain transcription factor signaling. (Adapted from: Sharrocks AD, Brown AL, Ling Y, Yates PR. The ETS-Domain Transcription Factor Family. *Int. J. Biochem. Cell. Biol.* 1997; **29**: 1371-1387).

1.4.1 Ets-1 and Ets-2

1.4.1.1 Function

Ets-1 and Ets-2 are expressed in a variety of cells, including endothelial cells (Ets-1 and Ets-2) and VSMCs (Ets-1). [26, 70, 71, 73, 75, 76, 80-90] Interestingly, although Ets-2 is postulated to be expressed ubiquitously, its expression in VSMC has not yet been proven. [71] The regulation of Ets-1 and Ets-2 is controlled to a great extent by the Erk-MAPK pathway.[26, 71-73, 75, 76, 81, 83, 84, 86, 90] Depending on which amino acid residues are phosphorylated, the activity of Ets-1 increases or decreases. Phosphorylation of Ets-1 at threonine-38, for example, activates DNA binding and target gene transcription, whereas phosphorylation of serine in the Ets-1 inhibitory domains prohibits DNA binding.[26, 87]

Under physiological conditions Ets-1 and Ets-2 play a critical role in the regulation of angiogenesis, apoptosis and the differentiation of myogenic, osteogenic, lymphoid and endothelial cell lines.[70, 71, 73, 75, 82] Evidence that Ets-2 plays a role in adult angiogenesis has, however, not been provided to date. [75] Furthermore, Ets-1 and Ets-2 play an important role in normal embryonic development. Mutations in Ets-1 and Ets-2 in mouse embryos result in defective vascular branching and consequently embryonic death around a gestational age of E10.5,[83] indicating an essential role of Ets-1 and Ets-2 in mouse embryonic development.[75, 76, 83] Homozygous Ets-2 $^{-/-}$ mouse embryos on the other hand did not survive beyond E8.5 as a result of inaccurate remodelling of the extracellular matrix and defects in the migration of trophoblastic cells.[73] Ets-1 and Ets-2 are also essential for the normal development of the coronary arteries and the myocardium in chicken embryos. [26, 87] Moreover, they are involved in VSMC proliferation in peripheral coronary arteries of chicken embryos.[91]

The target genes of Ets-1 and Ets-2 are numerous and mostly affect cell proliferation and differentiation, as well as angiogenesis and inflammatory reactions of the cellular environment. In VSMCs, Ets-1, for example, regulates transcription of PDGF A-chain and D-chain,[92, 93] PDGF-receptor α,[94] vascular endothelial growth factor (VEGF),[85] VEGF-receptor,[75] monocytes chemoattractant protein -1 (MCP-1),[26, 82] nicotinamide adenine dinucleotide phosphate **(**NAD(P)H) oxidase subunit p47(phox) (leading to increased ROS production),[95] Sm22α, [26] MMP-1, -2, [26] cyclin-dependent kinase inhibitor $p21^{CIP}$ (causing cellular hypertrophy) [82] and Fas ligand (FasL) (regulation of apoptosis).[71] In endothelial cells, Ets-1 affects the expression of endothelial nitric oxide synthase (eNOS),[73] $p21^{CIP}$ (causing cell cycle arrest),[82] vascular endothelial (VE)-cadherin,[70, 71, 73, 75] MMP-1, -3, -9 [70] and VEGF-receptors.[71, 73] The common target genes of Ets-2 include MMP-9,[73, 83] collagenase1,[73] cyclin D1,[73] VEGF-receptor Flt-1,[70, 73, 75] angiopoetin-2 (Ang-2), [75] cdc-2, [71] Bcl-xL [83] and p53.[73]

1.4.1.2 The role of Ets-1 and Ets-2 in disease

Ets-1 and Ets-2 are involved in the development of various diseases. An increase in transcriptional activity of Ets-1 and Ets-2 can induce enhanced cell proliferation, inhibition/upregulation of apoptosis, dedifferentiation, inflammatory reactions, vascular diseases, tumor-induced vasculogenesis, tumor invasion and even metastasis.[26, 50, 71, 76, 80, 82-85, 88, 90, 94, 95] This is caused either by overexpression at the gene level [71, 76] or by increased phosphorylation following stimulation by mitogens and cytokines (angiotensin-II, PDGF, ET-1, interleukin,[26, 82, 95-97] TNFα [26, 82, 86]) and reactive oxygen species (ROS).[92, 94]

Ets-1, for example, is involved in vascular remodelling after injury and mediation of vascular inflammation by promoting dedifferentiation, proliferation and migration of VSMCs activation of endothelial cells, extracellular matrix deposition and degradation [26-29, 70, 71, 80, 82, 84, 85, 88, 89, 94, 98, 99] and recruitment of inflammatory cells such as T-cells and monocytes.[26, 80, 82] These changes are known to occur specifically during development of intimal swelling and atherogenesis.[3, 18, 19, 26, 40, 41, 50]

Zahn et al., were able to show that in Ets-$1^{-/-}$ mice, expression of monocyte chemoattractant protein-1 (MCP-1) and cyclin-dependent kinase inhibitor $p21^{CIP}$ is significantly reduced in the presence of angiotensin II compared to the control mice, leading to a decreased recruitment of T-cells and macrophages as well as reduced uptake of leucine and thymidine in VSMCs. These findings indicate the important role of Ets-1 in mediating vascular inflammation and in promoting VSMC growth.[82] Angiotensin-II also leads to the Ets-1-dependent generation of ROS in VSMCs, thereby sustaining a vascular inflammatory condition.[95] Hydrogen peroxide (H_2O_2), on the other hand, functions as a mitogenic stimulus itself, activating Ets-1 and thus increasing PDGF-receptor α expression in VSMCs.[92, 94]

Kavurma et al., showed that there is a high level of Ets-1 expression in atherosclerotic lesions of human carotid arteries.[98] Furthermore, an involvement of Ets-1 activation in the progression and rupture of cerebral aneurysms due to increased expression of inflammatory

cytokines has been observed.[80] Overexpression of Ets-1 in VSMCs can also result in transcriptional repression of smooth muscle cell markers such as α-actin, MHC and SM22α, causing phenotypical switching to a more proliferative and less contractile phenotype.[50, 99] and inhibit apoptosis of VSMCs. [100]

Enhanced expression of Ets-1 is also found in several human cancer types including breast cancer, fibrosarcoma and hepatocellular carcinoma and is associated with a more aggressive behaviour of the tumor, including metastasis.[71] By regulating gene expression of matrix metalloproteinases (MMPs) and VEGF in VSMCs and endothelial cells, Ets-1 may promote tumor-induced angiogenesis and tumor invasion, [26, 85, 87-89] such as in hepatocellular carcinoma, as mentioned above.[71] In situ hybridization experiments showed transcription of c-Ets-1 in endothelial cells during tumor vascularisation.[88] Additionally, expression of Ets-1 in human ovarian carcinoma is associated with poor survival.[71]

As for Ets-2, its overexpression is found in two of the most common solid human cancer types, prostate and breast cancer. Moreover, it is essential for the malignant transformation of the lesional cells.[76] Ets-2 also has the ability to inhibit apoptosis in macrophages and mouse embryonic endothelial cells; this at least partially explains its role in tumorigenesis.[71, 72, 76, 83, 101]

1.4.2 Ets-2 repressor factor

1.4.2.1 Biology of Ets-2 repressor factor

Ets-2 repressor factor (Erf) was the first member of the Ets-domain transcription factor family to be identified as a repressor of gene transcription.[73, 76, 77, 102] It is a 75 kDa phosphoprotein consisting of 548 amino acids and ubiquitously expressed at a constant level in every type of tissue.[77, 102, 103] The homology with the other members of the Ets-domain transcription factor family is minimal, with the exception of the DNA-binding domain.[102, 103]

Erf is regulated via its subcellular localization.[76, 77, 103] In the unphosphorylated state, Erf binds to the promoter region of the ets-2 gene inside the nucleus, preventing its transcription. Upon activation of the Ras/Erk-1/2-MAPK pathway, Erf is phosphorylated and exported to the cytoplasm, exposing the DNA-promoter region of ets-2 which can now be transcribed, causing increased cell proliferation .[71, 73, 75-77, 102-104] Experiments with the Mek-1 inhibitor PD98059 were able to identify Erk-1/2 as the predominant regulator of Erf.[103]

However, Erf does not only mediate its anti-proliferative and growth-impeding effect via inhibition of ets-2 transcription; it also interacts with further growth and proliferation-stimulating genes such as c-myc and possibly c-fos by binding to their promoter regions.[77, 102-104] C-myc is a transcription factor with the ability to promote VSMC proliferation following activation by Erk-1/2.[37, 105] Regulation of c-myc transcription by DNA binding to the promoter region is dependent on multiple co-factors associated with Erf, such as E2F2, E2F4, E2F5 and the retinoblastoma protein(pRb) family.[104]

1.4.2.2 Nuclear shuttling of Erf

Extensive multisite phosphorylation by nuclear Erk-1/2 leads to the active export of Erf out of the nucleus.[76, 103] Export shuttling is dependent on chromosome region maintenance-1 (CRM-1).[77] Interestingly, the import of Erf into the nucleus can occur in both phosphorylated and unphosphorylated states, indicating that phosphorylated Erf can be shuttled bidirectionally.[77] Nevertheless, phosphorylated Erf is predominantly located in the cytoplasm and dephosphorylated Erf in the nucleus, as shown in rat embryonic fibroblasts.[77]

The change in subcellular localization of Erf occurs rapidly following adequate stimuli. Le Gallic et al., were able to demonstrate that 10 to 15 minutes after mitogenic stimulation, Erf was completely phosphorylated and relocated to the cytoplasm. However, following serum deprivation, all Erf was transported back to the nucleus within 30 to 45 minutes.[103]

1.4.2.3 Function of Erf

Erf is a transcriptional repressor and regulator of cellular proliferation and growth.[103] Embryonic death at 10.5 days post coitum in Erf $^{-/-}$ mice is suggestive of an essential role of Erf in mouse embryonic development and survival.[106] Experiments with phosphorylation-defective mutant Erf mouse embryonic fibroblasts were able to show that Erf induces cell cycle arrest by blocking progression of the cell cycle through G1 phase and arresting it in G0/G1 phase.[77, 102, 103] Moreover, it leads to suppression of Ras-induced tumorigenity.[103] A decrease in the repressing activity of Erf thus causes a higher risk of tumorigenesis.[72, 102, 103]

1.5 Aim of the study

Until now it is not known whether Erf protein expression occurs in VSMCs and how its phosphorylation is regulated.

Therefore we posed the following questions:

Is Ets-2 repressor factor expressed and/or phosphorylated in VSMCs?

Is Erk-1/2 an upstream regulator of Erf-phosphorylation?

Does Ca^{2+} regulate Erf-phosphorylation?

2. Materials and Methods

2.1 Materials, Chemicals and Antibodies

Complete growth medium used for cell cultures was high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS, 1% L-glutamine and 1% penicillin-streptomycin (PS). DMEM was purchased from Invitrogen Life Sciences (Switzerland). FCS, glutamine, PS and DMSO were obtained from Sigma-Aldrich (Switzerland).

The mitogen PDGF was obtained from Peprotech (UK) and the inhibitors BAPTA/AM, PD98059 and U0126 from Enzo Life Sciences (Switzerland). LDL was prepared in the laboratory.

The Bradford protein assay kit was obtained from Bio Rad Laboratories GMBH (Switzerland). The 8-10% SDS-PAGE gels were purchased from Invitrogen (Switzerland). Hybond ECL Nitrocellulose membranes, enhanced chemiluminescence (ECL) reagent and Hyperfilm^{TM} ECL were obtained from GE-Healthcare Life Sciences (Switzerland).

Goat anti-Erf and rabbit anti-total-Erk-1/2 antibodies were purchased from Santa Cruz Biotechnology Inc. (U.S.A.) Rabbit anti-phospho-Erk-1/2 antibody was obtained from Cell Signaling Technology (U.S.A.) and mouse anti-tubulin antibody from Sigma-Aldrich (Switzerland). All remaining reagents were purchased from Sigma-Aldrich (Switzerland).

2.2 Cell line and cultures

The cell line used for the experiments was the A-10 rat embryonic thoracic aorta vascular smooth muscle cell obtained from American Type Culture Collection (ATCC) USA. Cells were stored in cryoprotectant medium containing 5% DMSO in liquid nitrogen.

For use in the experiments, frozen vials of A-10 cells were carefully thawed in a water bath at 37°C and added to 4 ml warmed complete medium. Then the cells were centrifuged for 5 minutes at 1000 rpm and the supernatant was discarded. The remaining pellet was re-

suspended in 10 ml complete medium. 1 ml containing $1x10^5$ cells/ml was plated onto a 10 cm culture dish and 9 ml of complete medium added. The cells were then incubated at 37°C with 5% CO_2. Every three days the medium was changed and the cells checked under the microscope. At a confluence of 80% to 90%, the cells were either sub-cultured further or frozen in freezing medium (95% complete medium and 5% DMSO).

For sub-culturing the complete medium was removed and the cells were washed with 4 ml phosphate buffer saline (PBS) followed by addition of 1 ml trypsin. The cells were maintained at a temperature of 37°C for 10 minutes until detachment. Detachment was verified under the microscope and 4 ml of complete medium added. The cells were centrifuged again for 3 minutes at 1000 rpm. The supernatant was removed and fresh medium added. After careful suspension, 50'000 cells/ml were pipeted into each well of a 6-well plate for subsequent experiments.

2.3 Stimulation of A-10 cells

For the experiments, the cells were allowed to grow to approximately 80% -90% confluence in a 6-well plate. 24 hours prior to the experiment the cells were washed with 2 ml PBS and rendered quiescent by starvation overnight in 2 ml of starving medium (DMEM medium without FCS). On the day of the experiment, 30 minutes prior to starting, the 2 ml starving medium was removed from each well and replaced with 1 ml of fresh starving medium. The cells were left undisturbed in the incubator for 30 minutes at 37°C.

In order to analyze the effect of pro-mitogenic stimuli on the phosphorylation of Erf, the A-10 vascular smooth muscle cells were treated with PDGF or LDL. These mitogens are known activators of the MAPK pathway, as experimentally demonstrated by various other groups in the past.[24, 47-51, 107, 108]

The cells were pre-treated either with U0126 (10 µM) or PD98059 (25 µM) for 30 minutes or 45 minutes, respectively, at 37°C. [51, 107, 109-114] Control cells were treated with 0.1% DMSO

which was used to dilute the inhibitors U0126 and PD98059, for the same period of time. Then the cells were either stimulated with PDGF (10 ng/ml) or with LDL (50 ng/ml) for an additional 10 minutes at 37°C.[107, 108, 115, 116] After the incubation period, the medium was removed from the wells and 80 µl RIPA lysis buffer (Tris(hydroxymethyl)amino methane (Tris-HCl): 50 mM, pH 7.4; NaCl: 150 mM; ethylenediaminetetraacetic acid (EDTA): 1 mM; sodium deoxycholate: 0.25%; surfactant detergent NP-40: 1%; protease inhibitor cocktail (Complete mini Tablet, Roche, Switzerland); sodium orthovanadate (Na_3VO_4); phenylmethylsulfonyl fluoride (PMSF)) was added for 20 minutes to lyse the cells on ice. The lysates were then scraped from the wells, collected in eppendorf tubes and frozen in liquid nitrogen. Until further processing the samples were stored in the freezer at -25°C.

Mek-1/2-inhibitor PD98059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one], discovered by Dudley et al., is a selective inhibitor of the MAPK/Erk kinases 1 and 2 (Mek-1/2) with no significant influence on the MAPKs itself.[107, 112] As reported by Dudley et al. and confirmed by Favata et al., the IC_{50} for PD98059 is ~ 10 µM.[107, 109]

On the other hand, as Favata et al. showed, U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) is a highly-selective Mek-1/2 inhibitor with a 100-fold higher affinity for Mek-1/2 than PD98059. A concentration of 10 µM U0126 is sufficient for the 100% inhibition of Erk-1/2 phosphorylation. Half-maximal activity is reached at 1 µM U0126;[109] the IC_{50} of U0126 is 0.07 µM. Both inhibitors of the MAPK pathway are non-competitive inhibitors, probably of common or overlapping binding sites.[109]

To investigate the effect of intracellular calcium concentration in VSMCs, the calcium chelator BAPTA/AM was added.[24, 51, 68, 111] The cells were pre-incubated with BAPTA/AM (10 µM) for 45 minutes and the control cells were treated for the same period of time with 0.1% DMSO, which was used to dilute the inhibitor BAPTA/AM . During that time the plates were incubated at 37°C. Afterwards, the cells were stimulated with PDGF (10 ng/ml) for 10 minutes at 37°C and the subsequent steps were performed as described above.[107, 108]

BAPTA/AM is a potent intracellular Ca^{2+} chelator.[24, 68, 111] Previously, 20 µmol/l of BAPT/AM has been reported to be used in VSMCs.[68]

2.4 Protein estimation

The protein concentration was determined with the aid of the Bradford Protein Assay kit from Bio-Rad in accordance with the manufacturer's instructions.[117] Bovine serum albumin (BSA, 1 mg/ml) was used to make the protein standard series with concentrations of 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 (in µg/ml). Diluted lysis buffer was used as a control. The lysates were thawed and centrifuged at 13,200 rpm for 10 minutes at 4°C to separate the cell detritus from the proteins in the supernatant, which was transferred to fresh eppendorf tubes.

The samples (lysates) were diluted 10 times with water. Then 10 µl of the standards and 10 µl of the diluted samples were pipeted in duplicate into a 96-well plate. 200 µl of Bradford solution was added to each well and the plate was incubated for 5 minutes at room temperature. Protein concentration was determined using a photospectrometer (Bucher Biotec AG, Switzerland) at 595 nm. The sample concentrations were determined using the standard curve.

2.5 Western blotting

2.5.1 Protein separation

The proteins were separated by SDS-PAGE using 8-10% acrylamide gels. Before loading, the proteins were denatured by heating at 95°C for 5 minutes and centrifuged for 1 minute. Equal concentrations of protein were then loaded into each lane; the maximum volume was 20 µl. Protein marker mix containing proteins with defined molecular weights were also loaded on a separate lane. The gel was inserted in a chamber which was filled with running buffer (Tris-buffer: 25 mM, pH 10.5; glycine: 192 mM; SDS: 0.1%) and was exposed to a current of 80 V of direct current for 15 – 20 minutes followed by a current of 130 V for a total of 2 hours.

2.5.2 Protein transfer

The proteins separated by electric current were then transferred to a nitrocellulose membrane in transfer buffer (SDS-PAGE running buffer + 20% methanol) at 110 mA per membrane for a maximum of 2 hours. After the transfer, the membranes were checked for proteins with Ponceau S stain. The molecular weight of Erf is 75 kDa,[102] tubulin 55 kDa [118] and Erk-1 (p44) and Erk-2 (p42) 44 kDa and 42 kDa, respectively.[43, 119]

2.5.3 Antibody-staining

The membranes were washed and blocked for 1 hour with NET-gelatine buffer (NaCl: 0.15 M; EDTA: 5 mM, pH 8.0; Tris: 0.1 M, pH 7.5; Triton X-100: 0.05%; 2.5 g gelatine in 1 liter of water; final pH: 7.5), which was replaced every 15 minutes. Following initial blocking with NET-gelatine, the membranes were incubated overnight with the primary antibodies (anti-Erf or phospho-Erk-1/2) at 4°C, rocking gently. After washing again for 1 hour with NET-gelatine buffer to eliminate unbound antibodies, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-goat, anti-rabbit, and anti-mouse as required) for 1 hour, rocking gently at room temperature. The membranes were then repeatedly washed with NET-gelatine buffer for one hour at room temperature.

The protein detection and semi-quantitative analysis was carried out with the help of enhanced chemiluminescence (ECL).[24, 103, 111, 120] The membranes were incubated in 4 ml of the ECL reagents (2 ml of reagent A and 2 ml of reagent B) for 1 minute. The horseradish peroxidase (HRP) on the secondary antibodies oxidizes the luminol in the ECL reagents, leading to the emission of light that can be captured on film.[120] After washing with ECL, the blots were then placed in light-protected film cassettes together with the films (Amesham Hyperfilm™ ECL, GE Healthcare, UK). The exposure time varied from 30 seconds to 30 minutes to achieve a uniform strong signal on the film.

To detect if equal amounts of protein were loaded, the membranes were cleared of antibodies by stripping with 0.2 M NaOH for 15 minutes, followed by repeated washing for 2 hours in NET-gelatine buffer. Membranes were reblotted either with tubulin [121] or with total-Erk.[51]

2.6 Densitometry and statistical analysis

The developed blots were scanned (Epson Twain) and processed with Adobe Photoshop. Using the program NIH Image J, the density of the protein on the blots was measured individually for each lane and inserted into a Microsoft Excel file. The background was also measured for each lane and subtracted. Each set of experiments was performed 3 times and the mean values were calculated. The statistical analysis was carried out with the program StatView using the unpaired Students'*t*-Test. Data are given as means ± standard error of the means (S.E.M.). A P value < 0.05 was considered statistically significant. The unstimulated cells were taken as the 100% mark and used as the reference.

3. Results

3.1 PDGF-induced Erf phosphorylation is mediated by Erk-1/2

A-10 VSMCs treated with PDGF showed a 1.6-fold increase in Erf phosphorylation compared to unstimulated cells (**Fig. 6**; Unstimulated: 100% vs. PDGF: 159.9 ± 15.7%; *P*<0.05). In unstimulated cells, treatment with U0126 led to a reduction in basal Erf phosphorylation of 50% compared to the cells treated with 0.1% DMSO (0.1% DMSO: 100% vs. U0126: 53.9 ± 7.1%; *P*<0.05). Additionally, in cells treated with U0126 and PDGF, Erf phosphorylation was reduced by 3-fold in comparison with cells treated with PDGF only (PDGF: 159.9 ± 15.7% vs. U0126 + PDGF: 52.1 ± 11.2%; *P*<0.05). The level of Erf phosphorylation was similar in cells treated with U0126 in the absence or presence of PDGF.

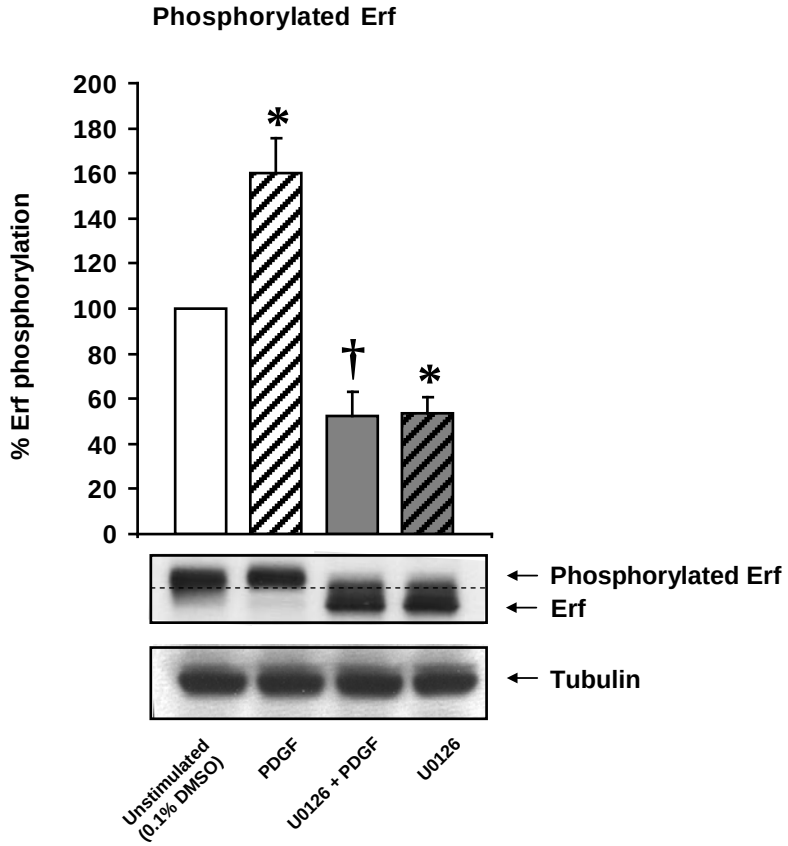

Fig. 6: Erf phosphorylation in VSMC. Cells were treated in the absence or presence of U0126 (10 µM) followed by treatment with PDGF (10 ng/ml). Unstimulated cells were treated either with 0.1% DMSO or U0126 (10 µM). Values represent percentage of Erf phosphorylation and are means ± standard error (n=3). * P < 0.05 vs. unstimulated, † P < 0.05 vs. PDGF. Values were compared using the unpaired t-test. The corresponding Western blots show the levels of phosphorylated Erf and total Erf. Protein loading was examined with tubulin. The dashed line on the Erf blot represents the cut-off used for differentiating between phosphorylated and unphosphorylated Erf.

In order to confirm that U0126 was indeed functional, we checked for the phosphorylation of Erk-1/2. The treatment of vascular smooth muscle cells (A-10) with PDGF led to a 4.4-fold increase in Erk-1/2 phosphorylation compared to unstimulated cells (**Fig. 7**; Unstimulated: 100% vs. PDGF: 439.0 ± 45.5%; $P<0.05$). The treatment of unstimulated cells with U0126 decreased the basal phosphorylation of Erk-1/2 by 6.6-fold compared to cells treated with 0.1% DMSO (0.1% DMSO: 100% vs. U0126: 15.6 ± 4.1%; $P<0.05$). There was a reduction in Erk-1/2 phosphorylation by 24-fold in the cells treated with U0126 and PDGF compared to cells treated with PDGF only (PDGF: 439.0 ± 45.5% vs. U0126 + PDGF: 18.4 ± 2.7%; $P<0.05$). The level of Erk-1/2 phosphorylation was similar in cells treated with U0126 in the absence or presence of PDGF.

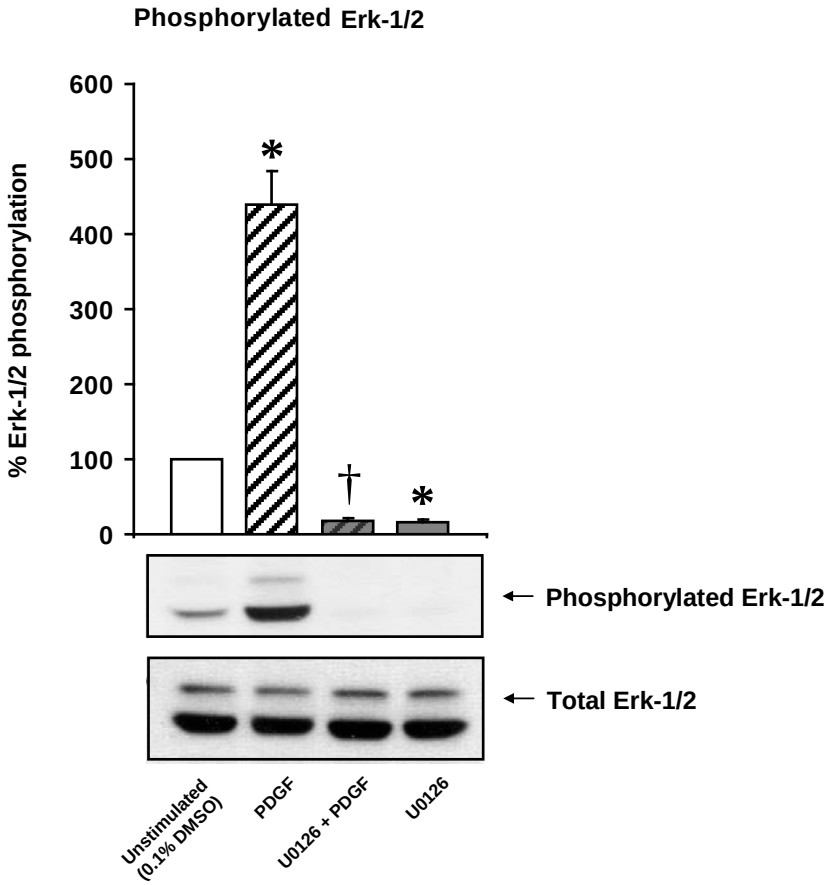

Fig. 7: Erk-1/2 phosphorylation in VSMC. Cells were treated in the absence or presence of U0126 (10 µM) followed by treatment with PDGF (10 ng/ml). Unstimulated cells were treated either with 0.1% DMSO or U0126 (10 µM). Values represent percentage of Erk-1/2 phosphorylation and are means ± standard error (n=3). ***** $P < 0.05$ vs. unstimulated, † $P < 0.05$ vs. PDGF. Values were compared using the unpaired t-test. The corresponding Western blots show the levels of phosphorylated Erk-1/2 and total Erk-1/2.

3.2 LDL-induced Erf phosphorylation involves Erk-1/2 activation

The stimulation of A-10 VSMCs with LDL led to a 1.4-fold increase in Erf phosphorylation in comparison to unstimulated cells (**Fig. 8**; Unstimulated: 100% vs. LDL: 137.5 ± 3.0%; *P*<0.05). Treatment of the cells with PD98059, followed by LDL, reduced Erf phosphorylation by a factor of 1.2 compared to treatment with LDL only (LDL: 137.5 ± 3.0% vs. PD98059 + LDL: 112.3 ± 4.9%; *P*<0.05). Compared to the cells treated with 0.1% DMSO, treatment with PD98059 led to a 1.3 fold reduction in basal Erf phosphorylation (0.1% DMSO: 100% vs. PD98059: 74.5 ± 4.4%; *P*<0.05).

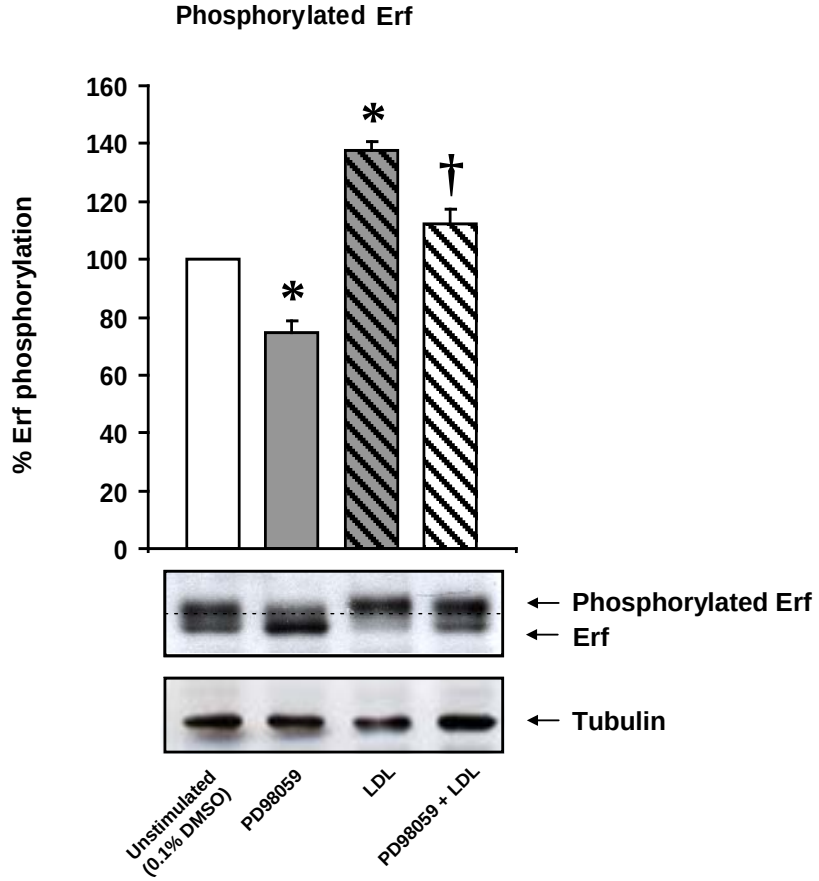

Fig. 8: Erf phosphorylation in VSMC. Cells were treated in the absence or presence of PD98059 (25 µM) followed by treatment with LDL (50 µg/ml). Unstimulated cells were treated either with 0.1% DMSO or PD98059 (25 µM). Values represent percentage of Erf phosphorylation and are means ± standard error (n=3). ***** P < 0.05 vs. unstimulated, † P < 0.05 vs. LDL. Values were compared using the unpaired t-test. The corresponding Western blots show the levels of phosphorylated Erf and total Erf. Protein loading was examined with tubulin. The dashed line on the Erf blot represents the cut-off used for differentiating between phosphorylated and unphosphorylated Erf.

To confirm that PD98059 was functional, it was necessary to check the status of Erk-1/2 phosphorylation. The application of PD98059 to the vascular smooth muscle cells led to a 3-fold reduction in basal Erk-1/2 phosphorylation compared to cells treated with 0.1% DMSO (**Fig. 9**; 0.1% DMSO: 100% vs. PD98059: 33.2 ± 8.7%; $P<0.05$). Stimulation with LDL caused a 1.8-fold increase in Erk-1/2 phosphorylation in comparison with unstimulated cells (Unstimulated: 100% vs. LDL: 179.4 ± 1.4%; $P<0.05$). Treatment of the cells with PD98059 and LDL caused Erk-1/2 phosphorylation to drop by a factor of 1.6 compared to the cells stimulated with LDL only (LDL: 179.4 ± 1.4% vs. PD98059 + LDL: 110.9 ± 14.7%; $P<0.05$).

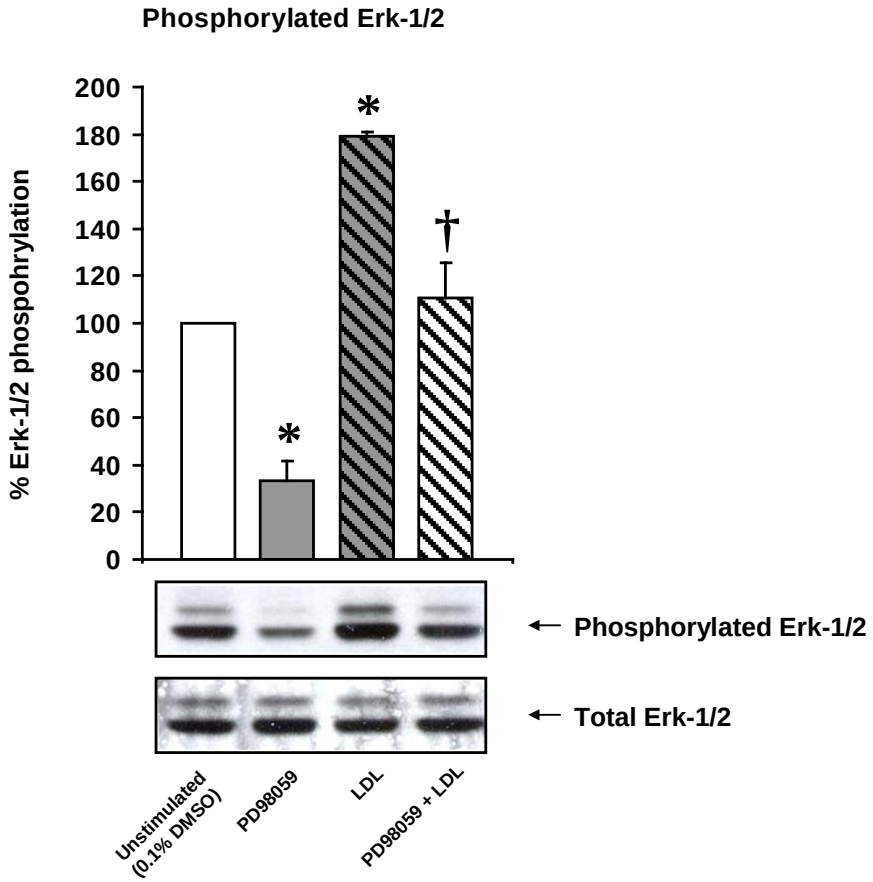

Fig. 9: Erk-1/2 phosphorylation in VSMC. Cells were treated in the absence or presence of PD98059 (25 µM) followed by treatment with LDL (50 µg/ml). Unstimulated cells were treated either with 0.1% DMSO or PD98059 (25 µM). Values represent percentage of Erk-1/2 phosphorylation and are means ± standard error (n=3). * $P < 0.05$ vs. unstimulated, † $P < 0.05$ vs. LDL. Values were compared using the unpaired t-test. The corresponding Western blots show the levels of phosphorylated Erk-1/2 and total Erk-1/2.

3.3 Erf and Erk-1/2 phosphorylation are mediated by depletion of intracellular Ca^{2+}

Vascular smooth muscle cells (A-10) stimulated with PDGF showed a 2.8-fold increase in phosphorylated Erf compared to unstimulated cells (**Fig. 10**; Unstimulated: 100% vs. PDGF: 277.9 ± 31%; $P<0.05$). Treatment of the cells with BAPTA/AM only increased Erf phosphorylation by a factor of 2.6 compared to the cells treated with 0.1% DMSO (0.1% DMSO: 100% vs. BAPTA/AM: 263.3 ± 22.4%; $P<0.05$). Treatment of the cells with both PDGF and BAPTA/AM resulted in a noticeable rise in Erf phosphorylation in comparison with the cells stimulated with PDGF only; the values, however, were not significant (PDGF: 277.9 ± 31% vs. PDGF + BAPTA/AM: 324 ± 12.4%; $P=0.24$). It was interesting to note that the level of Erf phosphorylation was similar in cells treated with PDGF or with BAPTA/AM.

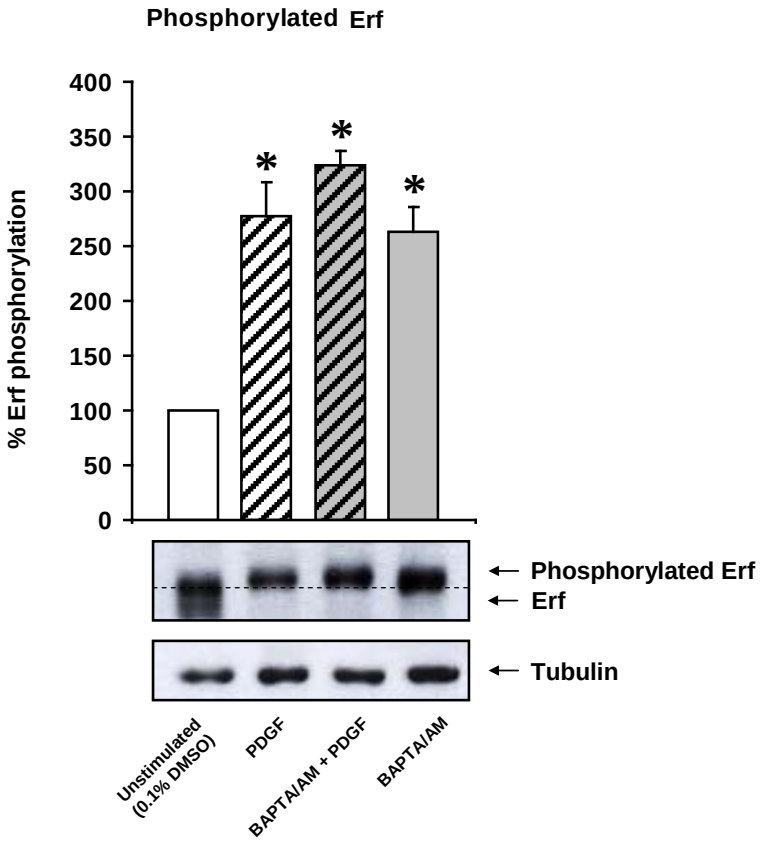

Fig. 10: Erf phosphorylation in VSMC following intracellular calcium depletion. Cells were treated in the absence or presence of BAPTA/AM (10 µM) followed by treatment with PDGF (10 ng/ml). Unstimulated cells were treated either with 0.1% DMSO or BAPTA/AM (10 µM). Values represent percentage of Erf phosphorylation and are means ± standard error (n=3). * $P < 0.05$ vs. unstimulated. Values were compared using the unpaired t-test. The corresponding Western blots show the levels of phosphorylated Erf and total Erf. Protein loading was examined with tubulin. The dashed line on the Erf blot represents the cut-off used for differentiating between phosphorylated and unphosphorylated Erf.

Whether calcium plays a regulatory role in mediating the phosphorylation of Erk-1/2 was also examined. The addition of PDGF to vascular smooth muscle cells (A-10) caused a 4.7-fold increase in Erk-1/2 phosphorylation compared to unstimulated cells (**Fig. 11**; Unstimulated: 100% vs. PDGF: 468.3 ± 19.6%; $P<0.05$). The treatment of unstimulated cells with BAPTA/AM also caused a 2.7-fold increase in Erk-1/2 phosphorylation compared to the cells treated with 0.1% DMSO (0.1% DMSO: 100% vs. BAPTA/AM: 268.3 ± 69.9%; $P<0.05$). Interestingly, stimulation of cells with both BAPTA/AM and PDGF potentiated Erk-1/2 phosphorylation by a factor of 4.1 compared to cells solely treated with PDGF (PDGF: 468.3 ± 19.6%, PDGF + BAPTA/AM: 1932.8 ± 69.9%; $P<0.05$).

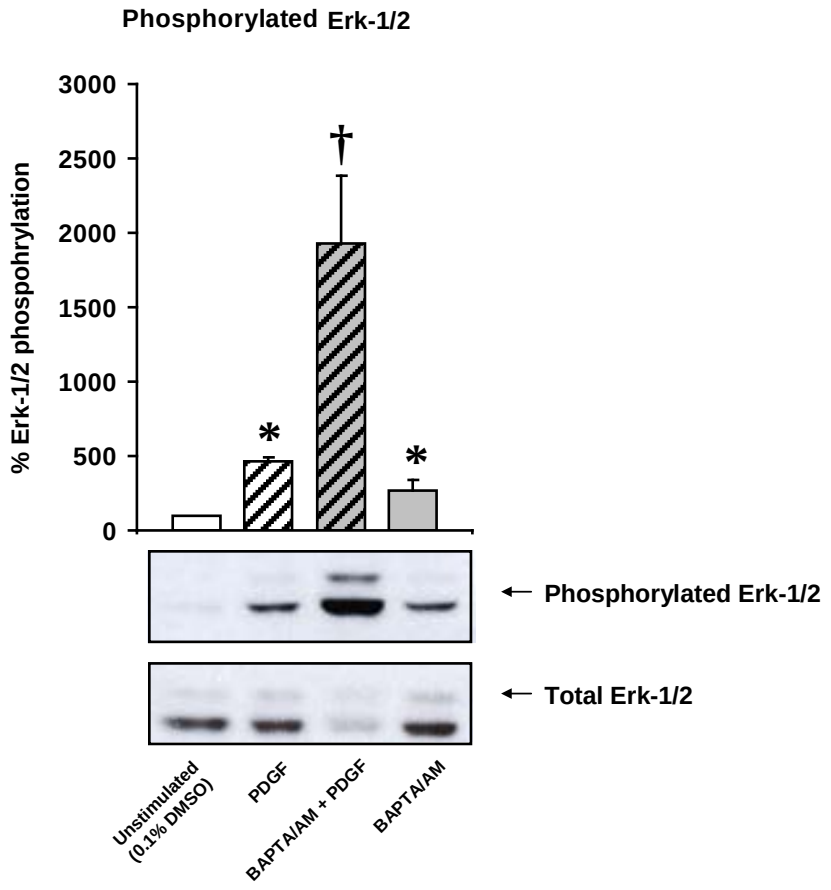

Fig. 11: Erk-1/2 phosphorylation in VSMC. Cells were treated in the absence or presence of BAPTA/AM (10 µM) followed by treatment with PDGF (10 ng/ml). Unstimulated cells were treated either with 0.1% DMSO or BAPTA/AM (10 µM). Values represent percentage of Erk-1/2 phosphorylation and are means ± standard error (n=3). * P < 0.05 vs. unstimulated, † P < 0.05 vs. PDGF. Values were compared using the unpaired t-test. The corresponding Western blots show the levels of phosphorylated Erk-1/2 and total Erk-1/2.

4. Discussion

4.1 Summary

In the present study the expression of endogenous Erf and its phosphorylation is shown to occur in VSMCs and the regulation of Erf phosphorylation is mediated by Erk-1/2 following mitogenic stimulation. Additionally, a decrease in intracellular calcium concentration, using an intracellular calcium chelator, acts as a mitogenic stimulus and increases phosphorylation of Erk-1/2 and Erf (**Fig. 12**).

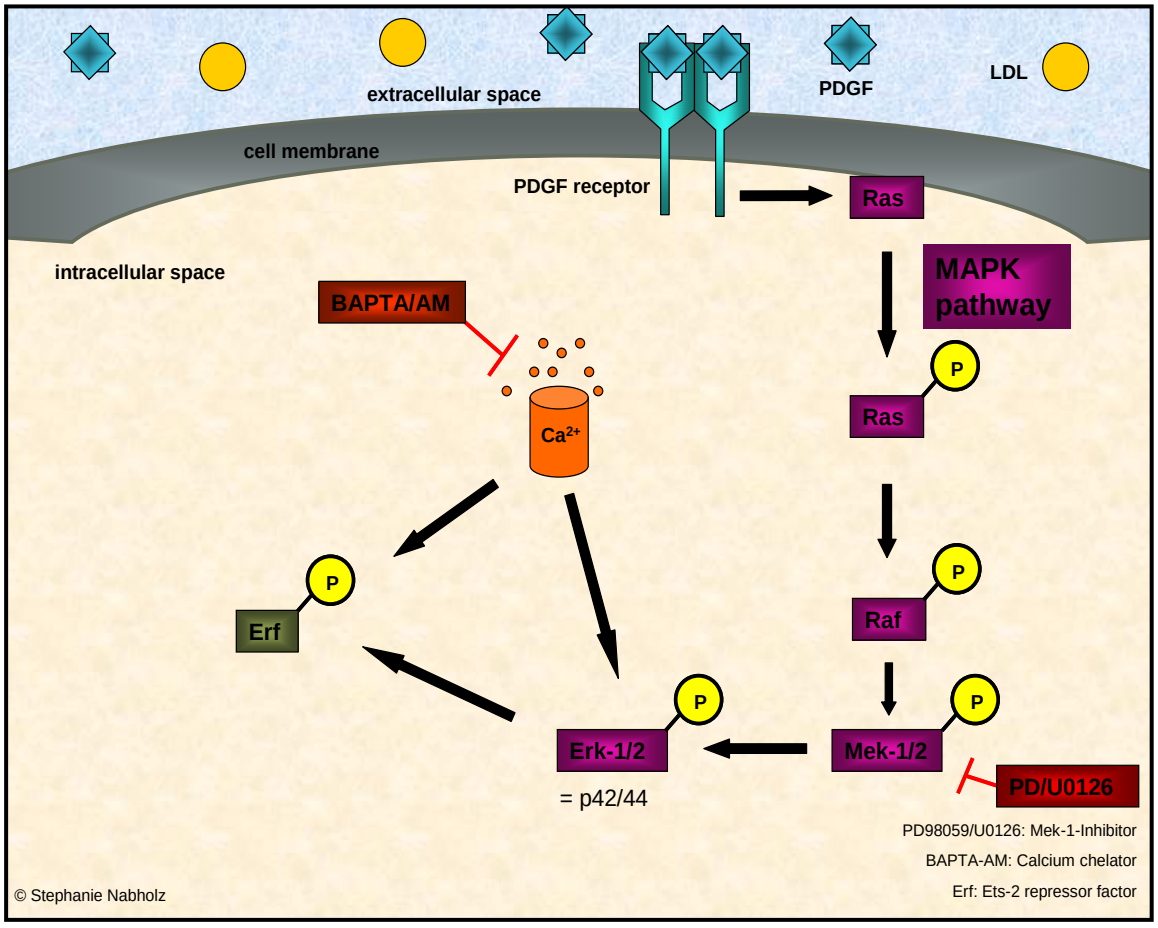

Fig. 12: Schematic diagram showing regulation of Erf phosphorylation by mitogens and $[Ca^{2+}]_i$. Mitogens (PDGF or LDL) bind to their cognate receptor. In this model, the PDGF receptor transfers the signal to the intracellular space by activating Ras, Raf and Mek-1/2. This signal further activates Erk-1/2, which in turn phosphorylates Erf. Additionally, BAPTA/AM reduces $[Ca^{2+}]_i$ by chelation of calcium, leading to induction of Erk-1/2 and Erf phosphorylation.

The experimental findings suggest that pro-atherogenic mitogens (such as PDGF or LDL) and the level of intracellular calcium activate Erk-1/2-dependent Erf phosphorylation, possibly

leading to VSMC proliferation, dedifferentiation and migration. Thus, phosphorylation of Erf may contribute to the pathogenesis of vascular diseases and its consequences.

4.2 Expression of Erf in VSMCs

The experiments on which the present study is based provide evidence for the expression of endogenous Erf in rat embryonic thoracic aorta VSMCs. This is the first study showing expression of endogenous Erf in VSMCs. Since VSMCs represent an important, integral element of the vasculature this as an important finding. However, it is still not known whether Erf is expressed in endothelial cells.

Under normal conditions, VSMCs are quiescent. However, during atherogenesis, driven by mitogenic stimuli, VSMCs migrate from the media to the intima, where they begin to proliferate, dedifferentiate and synthesize extracellular matrix components, causing intimal swelling and eventually atherosclerosis.[3, 18, 24, 26-28, 30, 31]

The Ets-2 repressor factor (Erf) belongs to a large family of transcription factors, the Ets-domain transcription factor family.[77] Consisting of numerous members, including Ets-1, Ets-2, Fli-1 and Elk-1, this transcription factor family regulates the expression of multiple target genes, mostly as a transcription activator.[26, 70-73, 75, 77, 102, 103] Erf however is an unique member of the family, since it acts as a transcription repressor.[77, 102, 103]

Previous studies on Erf have generally focused on cancer cells such as lung cancer cells, osteosarcoma cells (Saos-2), cervical cancer cells (HeLa), human teratocarcinoma cells (T2), human Ewing's sarcoma cells and v-Abelson leukemia virus transformed mouse leukemic monocytic cells.[77, 102, 103, 122-124] The expression of Erf has also been studied in mouse and rat embryonic fibroblasts, mouse myoblasts, liver tumor-infiltrating (TILs) CD8 T lymphocytes and even Drosophila S2 embryonic cells.[77, 103, 104, 125, 126]

The present finding, that Erf is also endogenously expressed in VSMCs, is interesting and may suggest its role in atherogenesis and the development of other angiopathies.

4.3 Phosphorylation of Erf is mediated by Erk-1/2 in VSMCs

Vascular smooth muscle cells are highly differentiated components of the vessel wall and found predominantly in the tunica media.[1, 2, 10] Here they contribute towards maintaining a constant blood flow in the large vessels of the body (Windkessel effect) and regulate organ perfusion and blood pressure.[2, 4, 6, 9-11] In the quiescent state, VSMCs proliferate at a very low rate and express specific markers such as smooth muscle α-actin, smooth muscle myosin heavy chains, myosin light chains, SM-22α, calponin, caldesmon and tropomyosin.[12]

Upon mitogenic stimuli with PDGF, LDL, angiotensin II or with reactive oxygen species (ROS) VSMCs start to proliferate and migrate to the intimal layer.[24, 48, 51, 52, 56, 57, 127-134] Chemotactic relocation is accompanied by a switch of the VSMCs from a quiescent phenotype to a proliferative, invasive phenotype, including gradual dedifferentiation, resistance to apoptosis and the loss of the specific contractile markers, especially smooth muscle α-actin, smooth muscle MHC, SM-22α, vinculin, caldesmon and desmin.[12, 18, 26-29, 50, 130, 135-139] The proliferative, dedifferentiated phenotype is furthermore characterized by an increased expression of matrix metalloproteinases and the phenotypic markers tropomyosin 4 (TM4) and myosin heavy chain embryonic (SMemb), as well as by the beginning of extracellular matrix (ECM) synthesis (glycosaminoglycan, collagen and elastin).[3, 18, 26, 130, 137, 140-142] Phenotypic switch of VSMCs, along with other processes, finally contributes to neointimal hyperplasia, the basis for vascular remodeling diseases (VRD) such as atherosclerosis or restenosis.[3, 12, 18, 26, 31, 50, 52, 54, 135, 142]

The predominant molecular pathway leading to the induction of the phenotypic switch in VSMCs is the mitogen activated protein kinase (MAPK) pathway.[26, 143, 144]. It regulates essential functions and processes of the cell such as proliferation, differentiation, apoptosis

and gene expression.[36-41] Present already in unicellular organisms, the members of the MAPKs are highly conserved serine/threonine kinases.[145] The best known and most important exponents of the MAPKs are the extracellular-signal-regulated kinases-1/2 (Erk-1/2), the Jnks and p38.[39]

In the present study, the experimental results suggest that in VSMCs, phosphorylation of Erf is controlled by Erk-1/2, as inhibition of Erk-1/2 by specific chemical Mek-1/2 inhibitors (U0126 or PD98059) reduced Erf phosphorylation significantly. The validity of Mek-1/2 inhibition was tested by checking the levels of Erk-1/2 phosphorylation. In cells treated with U0126, phosphorylation of Erk-1/2 was completely blocked, whereas Erf phosphorylation was decreased significantly, yet not abolished, suggesting that activated Erk-1/2 is not the only upstream mediator of Erf phosphorylation. Similar results were also obtained in the presence of PD98059, wherein the extent of inhibition of Erk-1/2 phosphorylation did not correlate completely with Erf phosphorylation. Again this is suggestive of the fact that other upstream effector molecules contribute to Erf phosphorylation besides Erk-1/2. The inhibitory influence of the Mek-1/2 inhibitor PD98059 was not as strong as U0126, especially after treatment with a mitogenic stimulus, confirming the lower affinity of PD98059 to Mek-1/2 than U0126. Similar data has been presented by Favata et al.[109]

In the present experiments VSMCs were stimulated with PDGF and LDL for a period of 10 minutes, leading to Erk-1/2 and Erf phosphorylation. This short stimulation of VSMCs can lead to cell migration.[40] Time-dependent stimulation experiments by Nelson et al., showed that mitogen induced MAPK activity of up to 15 minutes is necessary for cellular chemotaxis, whereas a MAPK activity of at least 1 and at the most 4 hours is required for VSMC proliferation.[40]

In its unphosphorylated state Erf is able to induce cell cycle arrest in the G0/G1-phase by blocking progression of the cell cycle through G1 phase,[77, 102, 103] therefore acting as an essential regulator of cellular proliferation and growth.[77, 102, 103] Sgouras et al., showed that in

mouse embryonic fibroblasts, phosphorylation of Erf is regulated via mitogenic stimulation and activation of the Ras/MAPK pathway and that phosphorylation of Erf inhibited its repressor activity.[102] Le Gallic el al., even identified Erk-1/2 as the predominant regulator of Erf in rat embryonic fibroblasts.[103] However, stress-activated protein kinases (p38 and Jnks) are not reported to induce phosphorylation of Erf.[103] Following phosphorylation by Erk-1/2 in the nucleus the repressor activity of Erf is lost and Erf is exported to the cytoplasm, revealing the DNA-promoter region of ets-2, c-myc and possibly c-fos as well as other proliferation inducing genes.[76, 77, 102-104] Enhanced transcription of c-myc and c-fos has been proposed to induce proliferation, migration and dedifferentiation of VSMCs.[86, 104, 105] In the present study, phosphorylation of Erf may also be associated with a decrease in Erf activity, accompanied by a higher rate of ets-2, c-myc and c-fos transcription and thus present a potential risk factor for the development of atherosclerosis and even tumorigenesis.[102-104]

4.4 The role of intracellular calcium in VSMCs

Calcium is an important regulator of different functions and processes of vascular smooth muscle cells.[58, 59, 146] Inside the cell, calcium is stored in caveolae along the plasma membrane as well as in the sarcoplasmatic reticulum.[1, 13, 58, 59, 65, 66] The binding of extracellular signals like adrenalin, angiotensin II, PDGF or LDL to membrane receptors followed by the activation of phospholipase C (PLC) and the formation of inositol-1,4,5-triphosphate (IP_3) trigger the release of calcium from the sarcoplasmatic reticulum into the cytoplasm through IP_3-receptor channels.[1, 58, 59, 62, 65, 66] Additionally, depolarization of the VSMC membrane activates the L-type high voltage-gated Ca^{2+}-channels located in the cell membrane, resulting in Ca^{2+} influx from the extracellular space.[58, 59, 65] Increasing intracellular calcium concentration ultimately activates calcium-dependent ryanodin receptor channels in the membrane of the sarcoplasmatic reticulum, causing further increase of intracellular calcium concentration.[58, 59]

The rise in intracellular calcium concentration causes VSMC contraction via calcium-calmodulin-dependent activation of the MLCK.[1, 8, 13, 16, 17, 58, 59, 65] Furthermore, calcium itself or the change in intracellular calcium concentration acts as a second messenger and induces Erk-1/2 activation in VSMCs.[68] Calcium is also known to regulate VSMC proliferation by activating the β-catenin/T-cell factor (TCF) signaling pathway via calcium-calmodulin-stimulated phosphodiesterase 1 (PDE1).[147] Blocking the rise of intracellular calcium concentration leads to down regulation of the lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) and the LOX-1 pathway activity, causing inhibition of VSMC apoptosis.[148]

In this study, depletion of intracellular calcium in VSMCs using 20 µM BAPTA/AM, alone or in combination with PDGF, activates Erk-1/2 phosphorylation and causes a subsequent increase in the phosphorylation of Erf. This is suggestive of the fact that Erk-1/2-mediated Erf phosphorylation is not only dependent on mitogenic stimulation but also on levels of intracellular calcium.

Kip et al., showed that a reduction of intracellular calcium concentration by 30% via chelation (20 µM BAPTA/AM) or antagonisation (20 µM Verapamil) of calcium increases Erk-1/2 phosphorylation and VSMC proliferation.[68] These findings are in line with our results. Using Ca^{2+}-channel deficient mice, they also demonstrated that animals develop vascular injuries based on medial wall thickening as a result of constantly reduced intracellular calcium concentrations.[68]

In contrast to our findings and the findings of Kip et al., Yang et al., showed that a concentration of 30 µM BAPTA/AM leads to a significant inhibition of oxLDL-induced Erk-1/2 phosphorylation in VSMCs.[24] Apart from that, they even postulated a mitogenic effect of calcium by examining VSMC proliferation.[24, 146]

These differences in findings concerning the pro- or anti-proliferative effect mediated by levels of intracellular calcium concentration may be due to 1) the method of reducing intracellular calcium concentration or 2) the cell systems used in experiments.

The present experiments additionally investigated the effect of a combination of mitogenic stimulus and calcium depletion on the A-10 VSMCs. The results showed that a cumulative impact on the cells caused a stronger phosphorylation of Erk-1/2 than each individual stimulus alone. These findings suggest that intracellular calcium levels mask the activation of MAPK pathway induced by mitogenic stimuli. However, the combination of PDGF and BAPTA/AM did not have the same strong effect on Erf phosphorylation as on Erk-1/2 phosphorylation. In VSMCs treated with PDGF and BAPTA/AM, phosphorylation of Erk-1/2 was significantly greater than in VSMCs stimulated with PDGF only (4.1-fold increase). In contrast, treatment with PDGF and BAPTA/AM resulted in a non-significant 1.17-fold increase in Erf phosphorylation compared to VSMCs stimulated with PDGF only. These findings indicate that not all of the information in Erk-1/2 is transmitted to Erf, thus supporting the notion that Erk-1/2 function as a further relay point to other downstream effector molecules.

The current results imply that, in combination with mitogens, the decrease of intracellular calcium concentration regulates Erf phosphorylation and represents a potential proliferative stimulus which may lead to alterations in vascular smooth muscle cell functions.[37-39]

4.5 Limits of the study

- In the present study, rat embryonic thoracic aorta smooth muscle cells (A-10) were used. It would be worth performing the experiments with other smooth muscle cell lines and also with primary vascular smooth muscle cells from mouse and rat.
- The role of Erf and its regulation by Erk-1/2 and calcium was not examined in intact arteries.
- Throughout the study only PDGF and LDL were used as stimulants. The effect of other agonists such as mitogens, inflammatory cytokines or reactive oxygen species on Erf activation was not investigated.
- Besides Erk-1/2 and calcium, other possible regulators of Erf phosphorylation were not studied. Furthermore, the localisation and nuclear shuttling of Erf in VSMCs was not analyzed.
- BAPTA/AM was used as a chelator of intracellular calcium. However, the level of intracellular calcium before and after BAPTA/AM application was not measured. Moreover, the role of other calcium chelators was not examined for the purposes of this study. Finally, the effect of BAPTA/AM on intact arteries was not taken into consideration.
- A further insight into the function of Erf in VSMCs could be achieved by the silencing of Erf expression at the gene level. This could provide knowledge about the role of Erf in cell proliferation, migration and differentiation.

4.6 Conclusions and clinical implications

Atherosclerosis and its sequelae coronary heart disease (CHD)/coronary artery disease (CAD) is the leading single most common cause of death in almost all of the Western industrialized countries (in Canada the leading cause of death in 2007 were malignant neoplasms).[149-152] In the United States in 2006, the prevalence of CHD was 9.1% in men and 7.0% in women. Accordingly, the prevalence for the total population (both sexes) was 7.9%. The death rate attributed to CHD in 2006 was 135/100'000.[150] In Europe 21% of the adult male and 22% of the adult female population died of CHD in 2008. However, death rates due to CHD are higher in Eastern and Central Europe than in Northern, Western and Southern Europe. In Switzerland for instance, the age-standardized death rate for CHD in 2004 was 20/100'000.[152]

According to a Swiss study by Faletra et al., coronary artery plaques (CAP) can be found in 49.9% of completely asymptomatic patients, whereby 18% of the patients have no cardiovascular risk factors whatsoever.[153] In the United States, the prevalence of coronary artery calcification is 17.6% in white men and 11.3% in white women in the 33-45 year-old age group and 70.4% in white men and 44.6% in white women in the 45-84 year-old age group.[154, 155] The data for developing countries such as India suggest a prevalence of 3-9% for coronary atherosclerosis in the general population.[151]

However, the mortality rate due to CHD and myocardial infarction (MI) has been declining. A community study in the United States showed a drop in the annual death rate due to CHD of 1.6% - 4.4%.[156] The overall reduction in CHD mortality for the United States from 1996 – 2006 amounted to 35.9%.[150] The first decline in CHD mortality in the United States was observed in 1975.[157] In Switzerland, the age-standardized death rates for CHD dropped from 60/100'000 in 1972 to 20/100'000 in 2005.[152]

Despite the decline in CHD and MI mortality, the risk factors, including arterial hypertension, nicotine abuse, diabetes, hypercholesterolemia, obesity and lack of sufficient physical

exercise are widely spread and reaching pandemic proportions - so far, prevention has been the only effective therapy.

Exploring the mechanisms of atherogenesis is a major field in medical science because it affects the survival of an increasingly aging population, particularly in Western countries. However, another reason for the great interest in this field is that pharmaceutical companies are willing to spend considerable amounts on scientific findings that will enable them to develop drugs that will allow the Western civilization to maintain its unhealthy and decadent lifestyle.

Although a major part of the molecular processes in VSMCs during atherogenesis is still not completely understood, research in this field has shown that VSMCs play a central role. Furthermore, data indicate that the MAPK pathway is critical to VSMCs in the development and progression of atherosclerosis.

In the last few years the MAPK pathway has gradually become the center of attention in cancer research.[37-41, 158, 159] Biochemical mutation and activation analysis have provided evidence that in a variety of types of cancer, the MAPK pathway, and especially the Ras-Raf-Mek-Erk-1/2 pathway, is constitutively overactivated.[158, 159] The underlying mechanisms leading to this condition are manifold, although the most common is the mutation of a member of the MAPK pathway. In colorectal cancer, for example, mutational analysis studies were able to identify b-Raf (V600E) mutations in 15.6% and k-Ras mutations in 22% of the cases.[160] The presence of b-Raf mutations is associated with a poor prognosis of survival and a higher tumor grade, whereas mutations of the k-Ras gene have no impact on overall survival or progression-free survival but provide additional information for appropriate treatment with tyrosine kinase receptor inhibitors (TKIs).[161-167] Thus, the mutational statuses of k-Ras and b-Raf represent prognostic and predictive factors of colorectal cancer.[165] Mutation of k-Ras in non-small cell lung cancer (NSCLC) in contrast is a predictor of a poorer clinical outcome.[168]

Not only mutations of the MAPK pathway can induce its overactivation; mutations upstream of the cascade mediate the same effect.[158, 169, 170] For example mutations of epidermal growth factor receptor (EGFR), a tyrosine kinase receptor, occur in approximately 13% of non-small cell lung cancers (NSCLC) and are associated with a survival benefit and better response to tyrosine kinase receptor inhibitors (TKI) as well as to chemotherapy.[168, 171] However, when an EGFR mutation occurs simultaneously with a k-Ras mutation, the response to TKIs is much weaker.[168, 171]

The formation of fusion genes through translocation of chromosomal regions can also lead to overactivation of the MAPK pathway.[71] The best known translocation is probably the t(9;22) translocation, resulting in Philadelphia chromosome 22.[158, 172] Its gene product is the fusion-protein Bcr-Abl, a constitutively activated tyrosine kinase that causes increased activation of the MAPK pathway by permanent phosphorylation of Ras and Raf.[173-175] The t(9;22) translocation is found in almost all cases of chronic myeloid leukemia (CML), however, according to recent studies, it is probably not the only translocation and translocation is not the only reason for malignant transformation of the hematopoietic cells.[158, 172, 176, 177]

Growing insight into the role of the MAPK pathway in different types of solid and hematopoietic cancers has led to the development of targeted agents, constructed to attack the specific properties of tumor cells. Screening for designated mutations depending on the origin of the malignant tissue provides the necessary information for individualized therapy.[167] One of the most widely applied classes of targeted cancer therapeutics are the so called small-molecule tyrosine kinase inhibitors (TKIs), monoclonal antibodies against activated tyrosine kinases of different cancer types.[167, 168, 172, 178-183] The best known examples are the Bcr-Abl kinase inhibitor Imatinib (Glivec®), used in the treatment of Philadelphia-positive CML,[184] epidermal growth factor receptor (EGFR) inhibitor Trastuzumab (Herceptin®), used in the treatment of human epidermal growth factor receptor-2/neu (Her-2/neu)-positive breast cancer [183] and the EGFR inhibitor Erlotinib (Tarceva®), used in the case of EGFR-mutation

positive NSCLC.[180] However, inhibitors of Ras, b-Raf and Mek-1/2 are also currently being tested as cancer therapeutics.[179]

The fact that the MAPK pathway is ubiquitously expressed and plays a key role in a variety of intracellular processes, as well as in the development of human cancer, implies that it could also be involved in the development of other diseases.

Thus, as a next step, our knowledge of the MAPK pathway and receptor tyrosine kinase mutations, as well as fusion-protein formation activating the MAPK pathway during tumorigenesis, should be adapted to the model of atherogenesis. VSMC proliferation, dedifferentiation and migration during intimal hyperplasia formation are the response to a mitogenic stimulus. Up until now it has been assumed that these events are triggered by an external signal. However, considering the most recent discoveries in cancer research, an endogenous, mutational origin of these changes is conceivable, especially in patients prone to atherosclerosis. Future studies should focus on answering the question whether mutations or translocations might possibly be the cause of atherosclerosis.

The drugs currently available on the market for the therapy of atherosclerosis and CHD/CAD focus on fighting the risk factors of atherosclerosis such as hypercholesterinemia (lipid-lowering agents, statins) and hypertension (ACE-inhibitors, beta-blockers), or on preventing acute thrombus formation (thrombocyte aggregation inhibitor, Aspirin). None of these agents are targeted to attack the essential processes of atherogenesis. Thus, taking the fact into consideration that the MAPK pathway is critical in the regulation of VSMCs during atherogenesis, it would be worth investigating the effects of TKIs on the development and progression of atherosclerosis.

Several members of the Ets-domain transcription factor family are also activated excessively in the course of mutational overactivation of the MAPK pathway. However, the Ets-domain transcription factors can also directly acquire constitutive activity through gene mutation or chromosome translocation with protein fusion. The resulting deregulation of the Ets-domain

transcription factors then leads to uncontrolled proliferation of the lesional cell and finally development of solid cancer and leukemia. [70-72, 74-76, 85, 87-89, 185-188]

In breast cancer, Ets-1 is overexpressed and is consequently involved in the progression of the disease and metastasis. The invasive behaviour of this type of cancer, however, is not only mediated by the overexpression of Ets-1 in the tumor cells, but also by overexpression in the endothelial cells and VSMCs of the blood vessel supply.[70, 71, 75, 87-89] Thus, Ets-1 has become a prognostic marker for poor survival prospects in connection with breast cancer.[87, 89]

The transcription factor Ets-2 is also associated with certain types of human cancers. Hsu et al., were able to show that in two of the most common types of solid malignomas, breast and prostate cancer, there is a crucial rise in Ets-2 expression.[76] In the course of malignant transformation, activated Ets-2 increases the level of human telomerase reverse transcriptase (hTERT) expression, thereby decreasing the apoptosis rate of cells with damaged DNA.[26, 188]

However, Ets-1 and Ets-2 do not only play a crucial role in tumorigenesis and cancer progression; they are also essential for normal development of the mouse and chicken embryonic vascular system.[26, 75, 76, 83, 91] Also, Ets-1 is known to be involved in human angiogenesis as well as tumor vascularisation, vascular inflammation, rupture of cerebral aneurysms, and finally, atherogenesis.[26, 70-73, 75, 80, 82, 85-89, 98]

In analogy to the MAPK pathway the knowledge of Ets-1 and Ets-2 mutations in human cancer could be applied to the model of atherosclerosis. Possible mutation/s-associated overactivation of Ets-1 and Ets-2 during atherogenesis could be a potential field to investigate. Various groups have been able to show participation of Erf in the pathomechanisms of disease development in different cell systems as well as in the carcinogenesis of various cancer types due to a downregulation of Erf transcription and repressor activity.[122, 123, 125]

In lung cancer, the microRNA miR-7, whose expression is induced by an activating mutation of epidermal growth factor receptor (EGFR) and subsequent upregulation of the Ras/Erk pathway, decreases the level of Erf by direct targeting. As a consequence, growth and proliferation of the lesional cell is increased, finally causing tumorigenesis.[122] Hester el al., provide evidence that Erf negatively regulates the cell cycle genes c-myc and cdc2, and thereby affects cell cycle progression and proliferation. Moreover, they demonstrate that constitutive activation of the MAPK pathway by v-Abelson leukemia virus-transformed mouse leukemic monocytic cells causes constitutive inactivation of Erf repressor activity, suggesting that Erf is a specific target for inactivation by oncogenes in the process of malignant transformation.[123]

On the other hand, overexpression of Erf can also be the cause of disease development. In Duchenne muscular dystrophy (DMD) it represses the expression of utrophin, a homologue of the defectively or non-expressed dystrophin gene, and potentially contributes to disease deterioration.[126]

Prior to this study little was known about the expression of Erf in VSMCs and still nothing is known about the target genes of Erf and how Erf regulates transcriptional repression in VSMCs, with the help of co-factors. Although an enhanced Erf phosphorylation is known to cause ets-2 and c-myc deregulation in mouse embryonic fibroblasts and thus lead to increased tumorigenesis,[102, 104] the question remains as to under which conditions in VSMCs Erf is over-phosphorylated and thus looses its protective function, thereby allowing enhanced ets-2 and c-myc transcription. The answer to that question could be of major relevance in atherosclerosis research.

The present study shows that Erf is phosphorylated extensively via the Mek/Erk-1/2 MAPK pathway in VSMCs following mitogenic stimulation. This novel finding could help us understand the development of the various diseases of the vessel wall including atherosclerosis, and thus help in the development of preventative or therapeutic agents. For

example, the targeted activation of Erf transcription or inhibition of Erf phosphorylation could lead to the prevention or attenuation of VSMC proliferation, dedifferentiation and migration to the intima during intimal thickening. In this way atherosclerosis and its far-reaching consequences could be treated effectively or even prevented from developing. In addition, quantitative measurements of phosphorylated Erf in VSMCs, perhaps within a puncture of the arteria radialis, could contribute to the development of a risk-stratification score for atherosclerosis.

Based on the results emerging from this work an investigation of the effect of an aimed increase in $[Ca^{2+}]_i$ in VSMCs on proliferation, migration and apoptosis or even on the development and progression of atherosclerosis could represent the next step towards the development of therapeutic or preventative drugs. Furthermore, due to our findings in relation to calcium concentrations, studies suggesting the use of calcium channel blockers (CCB) as a potential additive in the treatment of atherosclerosis and other vascular diseases should be reconsidered.[189, 190]

In the last few years a new type of targeted therapy involving micro-RNAs (miRNAs) against vascular disease and phenotypic switching of VSMCs has been increasingly gaining in importance. These ubiquitously-expressed specific non-coding ribonucleic acids regulate translation of their particular target RNA post-transcriptionally.[191-195] Special attention has been given to the miRNA miR-145, which is physiologically expressed at a high level in VSMCs of healthy arterial walls and positively regulates the expression of VSMC markers such as SM α-actin and MHC.[191, 195] Zhang et al., showed that the expression of miR-145 is downregulated significantly in VSMCs of balloon-injured arteries with neointimal hyperplasia and dedifferentiated VSMCs.[191, 195]

In the future miR-145, as well as other micro-RNAs, could become the novel markers of VSMC phenotypic switching and starting point for therapeutic approaches to vascular remodeling diseases.[191-195] By directing the focus towards the development of Erf-enhancing-

miRNAs, phosphorylation of Erf in mitogenically-challenged VSMCs could be compensated, allowing continued repression of ets-2 and c-myc transcription and blockage of cell cycle progression and neointimal formation.

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7. Curriculum vitae

Personal Data

First Name, Name	Stephanie Jane, Nabholz
Date of Birth	June 1st, 1984
Place of Birth	Kilchberg, ZH
Town of Citizenship	Zürich, ZH
Nationality	Switzerland/Canada

Education

1991 – 1997	Primary School in Wettswil am Albis, ZH
1997 – 2003	High School in Kantonsschule Wiedikon, ZH (Matura Type: Altsprachliches Profil)
2003 – 2009	Studies of Human Medicine, University of Zürich
September 2009	Swiss Federal Medical Exams (Staatsexamen), University of Zürich

Professional occupation

2009 – 2010	Assistant Doctor in Obstetrics and Gynaecology, Kantonsspital Baar, ZG, Chief physician ad interim Dr. med. Susanna Harlacher
Since 2010	Assistant Doctor in Pathology, Kantonsspital Baden, AG, Head of department Prof. Dr. med. Gad Singer
Since 2006	Basic research in Vascular Biology at Research Unit Internal Medicine, Clinic and Polyclinic of Internal Medicine, University Hospital Zürich, Clinical Director Prof. Dr. med. Edouard Battegay